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SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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This issue in two Parts

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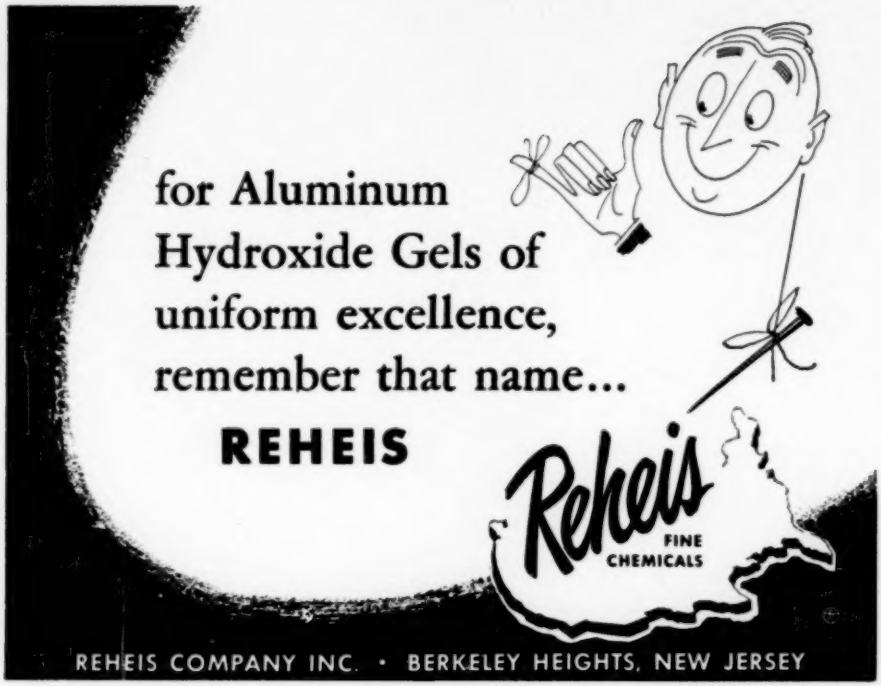

NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in Buffalo, New York, August 26 to September 1, 1951. Members planning to present papers before any Section during this meeting are earnestly requested and strongly urged to notify the appropriate Section secretary of their intention promptly. Titles and abstracts (not to exceed 250 words) of papers should be submitted by June 1, 1951 or preferably earlier, in order to insure a place on any Section program. Send titles and abstracts of papers to:

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This issue of the *Scientific Edition* of the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION is supplied in two parts. Part I is the customary edition of the JOURNAL, and Part II is a supplement carrying the list of active members of the AMERICAN PHARMACEUTICAL ASSOCIATION, the roster of committees and delegates, a brief history and summary of the ASSOCIATION's activities, the list of officers of the ASSOCIATION and places of meeting since its organization, the Constitution and By-Laws, the Certificate of Incorporation, the Code of Ethics, the lists of National, state and local pharmaceutical organizations with the names of their Secretaries, the list of accredited Colleges of Pharmacy, State Boards of Pharmacy, and other similar information.

Prior to 1945, the PROCEEDINGS of the AMERICAN PHARMACEUTICAL ASSOCIATION were published either separately or in installments in the monthly JOURNAL. Since 1949, we have been issuing the information referred to in the previous paragraph as a supplement to the *Scientific Edition* of the JOURNAL so that it could be available as a separate volume to those who refer to this information frequently. The PROCEEDINGS of the Annual Convention are published in narrative form in the *Practical Pharmacy Edition* of the JOURNAL immediately following the annual convention.

The PROCEEDINGS of the 1950 convention were published in narrative form in the May number of the *Practical Pharmacy Edition* of the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Later editions of the JOURNAL carried the addresses and reports of special interest. The scientific papers and those dealing with practical pharmacy, economics, history, education, and legislation which were presented at the various section meetings are expected to be published in various issues of either the *Scientific* or *Practical Pharmacy Edition* of the JOURNAL.

In addition, there was made available to the officers and members of the House of Delegates of the ASSOCIATION, to active members desiring it, and to libraries, a condensed stenographic transcript of the general sessions and the sessions of the House of Delegates at the 1950 convention, omitting the addresses and reports published in the JOURNAL. Copies of these PROCEEDINGS are still available to members of the ASSOCIATION without charge, and to non-members at $2.00 per copy.

The membership list, which is a part of the supplement to the January, 1951, *Scientific Edition*, includes active members in good standing as of October 31, 1950. Members who joined the ASSOCIATION after October 1, 1950, have been listed in one of the issues of the *Practical Pharmacy Edition* of the JOURNAL appearing since that date, and their names will, of course, be included in the next supplement to be published in January, 1952.

Members of the ASSOCIATION are earnestly requested to check the accuracy of the address given with their names and send us any necessary changes.

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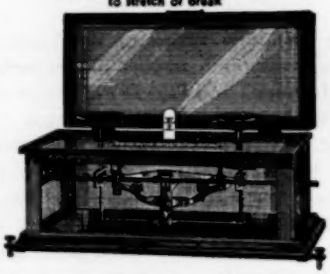

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Robert P. Fischelis, *Secretary and General Manager*

NOTICE TO AUTHORS OF PAPERS

Manuscripts.—All manuscripts submitted to the Editor or presented to the Sections of the ASSOCIATION should be ready for publication, typewritten with double spacing on one side of good quality paper 8.5″ x 11″ in size and with 1″ to 1.5″ margins. In addition to the original typewritten copy one or more carbon copies should be submitted in order to facilitate examination by members of the Editorial Advisory Board or other referees. Each manuscript should be accompanied by an abstract of not more than 250 words including a summarization of conclusions and recommendations. All pages in manuscripts, including tables but not figures, should be numbered consecutively. Previously published and readily accessible procedures which have been followed in experimental work should not be made a part of the manuscript, but should only be referred to by appropriate literature references.

Form of Presentation.—Titles of manuscripts should be kept as short as possible and preferably restricted to one line. The name of the laboratory in which the work described was done should be placed in a footnote at the end of the first page. Each manuscript should begin with one or more introductory paragraphs indicating the intention and meaning of the investigation and the method of procedure. The introduction should be followed by a report of the essential details of the experimental work, and a discussion or interpretation where necessary. Finally there should be a brief summary of conclusions and recommendations. Authors may find it helpful to arrange manuscripts to conform to the style of presentation as illustrated in papers on analogous subjects published in recent issues of THIS JOURNAL.

In preparing manuscripts for publication in THIS JOURNAL center headings should be avoided so far as possible. Side headings followed by a period and dash should be indented and run into the part of the text to which they apply. Such side headings may be used as freely as desired. Literature citations should be numbered consecutively in order of appearance in the manuscript and should be indicated by full-sized Arabic numerals enclosed in parentheses. If a literature reference is cited more than once in a manuscript subsequent citations should be indicated by the first number assigned. References to footnotes throughout the text should be numbered consecutively by superscript Arabic numerals, but such references in each table should be designated by superscript lower case letters beginning with "a" and continuing in natural order. Literature citations should be grouped at the end of the manuscript under the heading "References." The citations should be numbered consecutively with full sized Arabic numerals enclosed in parentheses corresponding to the numbers used in the text. The names of all periodicals cited in the list of references must be abbreviated in accordance with abbreviations given by *Chemical Abstracts* in its "List of Periodicals Abstracted." The sequence followed in the citations must be as follows:

(1) Doe, J. B., *Am. J. Physiol.*, 79, 289 (1927).

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(1) Gilman, H., "Organic Chemistry," ed. 2, John Wiley and Sons, New York, 1943, vol. 2, pp. 1236, 1252.

Arabic numerals must be used to designate all definite weights, measurements, percentages and degrees of temperature unless occurring at the beginning of a sentence, in which case the numeral and measurement are spelled out. Periods of time should be written in words. Unusual characters and Greek letters should be written plainly and legibly or should be explained by notes on the margin of the page. Fractions should be written with a slant line. Standard abbreviations should be used whenever weights and measures are stated in the metric system, e.g., 10 Gm., 2.5 cc., etc. The forms to be used are: cc., Kg., Gm., mg., mm. cm., L. and M.

Figures.—All drawings should be made in India ink, preferably on white tracing paper or cloth. Original drawings are preferable, but clear, well-prepared glossy photographic prints are acceptable. The size of illustrations should not exceed the 8.5″ x 11″ manuscript size. They should be designed for reduction to the width of one column (2.75″) or full page width (5.5″). Thus, height rather than width should be accentuated where possible.

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Tables.—Tables should be carefully constructed so that the data presented may be understood easily. The indiscriminate use of tables should be avoided. Tables should be so constructed as to occupy fully a single column (2.75″) which will take about 40–50 characters and spaces. Tables which require two columns are also acceptable. Interpretive statements should not be written on the body of a table but should be explained in footnotes. Column heads should be made as small as possible and standard abbreviations should be used liberally. Data which may easily be deduced by simple arithmetic from data in another column should not be included. All tables should be referred to in the text of the manuscript and the author should indicate as nearly as possible the positions in which the tables should be set. It is not necessary to place tables on separate sheets, and in fact it is preferable to intersperse them in the manuscript. Tables should be numbered consecutively using Roman numerals. The table number and the title should be placed in a continuous heading above the data presented. The preparation of a clear and concise table of data requires much planning and infinite care.

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Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XL — *JANUARY, 1951* — NUMBER 1 — CONSECUTIVE NO. 2

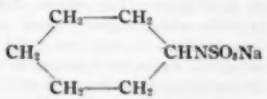
Studies on Cyclamate Sodium (Sucaryl Sodium), A New Noncaloric Sweetening Agent*,†

By R. K. RICHARDS, J. D. TAYLOR, J. L. O'BRIEN, and H. O. DUESCHER

While saccharin has proved over the years to be an innocuous noncaloric sweetening agent, its instability to heating and bitter aftertaste have been distinct drawbacks. A new chemical substance, cyclohexyl sulfamate sodium (Sucaryl sodium), is less potent in sweetening power than saccharin, but withstands boiling and has a clean sweet taste. Pharmacologic studies indicate this substance to be nontoxic in acute and chronic experiments in various species. It does not exert specific physiological effect. Sucaryl sodium is excreted largely unchanged by the kidneys, has no effect upon oxygen consumption, and does not interfere with the digestive enzymes. The presence of Sucaryl sodium in the urine does not disturb the usual chemical tests or cause false positive results.

SINCE the discovery of saccharin by Remsen and Fahlberg in 1897 (1), this sweetening agent has remained in its dominant position as a sugar substitute, especially for the diabetic patient and for dieting purposes. Its harmlessness, if used for these purposes, is well attested by animal studies as well as by many years of clinical experience. Two drawbacks of saccharin are its somewhat bitter aftertaste and its instability, if exposed to heat or acid solutions.

During chemical studies of organic sulfamates, Dr. Michael Sveda, working in the chemical laboratory of the University of Illinois, discovered the sweetening properties of a compound entirely unrelated chemically to saccharin, namely sodium cyclohexylsulfamate. The structural formula is as follows:



The sodium salt, which is now being introduced under the name Sucaryl sodium (cyclamate sodium, Abbott), is a white crystalline powder readily soluble in water and having a clean, sweet taste in concentrations up to 0.8%. In more concentrated solutions, a bitter aftertaste becomes noticeable. Solutions of Sucaryl sodium are practically neutral, and are resistant to boiling under the acid or alkaline conditions usually occurring in the preparation of foodstuffs. Comparison of its sweetening activity under controlled conditions which were conducted by the

* Received August 11, 1950, from the Depts. of Pharmacology and Analytic Chemistry, Abbott Laboratories, North Chicago, Ill.

† Sucaryl sodium is the registered trade mark name of Abbott Laboratories, North Chicago, Ill., for cyclamate sodium.

pharmaceutical department of Abbott Laboratories, showed that Sucaryl sodium is 30 times sweeter than sugar, as compared with 400 for saccharin. Clinical tests with Sucaryl sodium indicate that it is readily accepted by the patients, many of whom comment spontaneously on the freedom from aftertaste. Its stability to acids and boiling would open possibilities for its future use in canning and other preparations of special foods in which a sweet taste but the absence of sugar is desired. In the following, a concise description of some of the extensive studies which have been conducted in our laboratories is presented, as well as some data on the excretion of Sucaryl sodium after oral administration to the human.

ACUTE TOXICITY STUDIES

Acute toxicity determinations were conducted on various species, using several routes of administration. It was found that the LD_{50} after oral administration in mice lies between 10 and 12 Gm./Kg. Death occurred one to twenty-four hours after administration, and post-mortem examination showed fluid accumulation in the intestine, the lungs appearing normal, and the heart being slightly distended. In the same species, the LD_{50} by intravenous administration was found to be 4 Gm./Kg. Death occurred within a few minutes after injection, preceded by clonic convulsions and coma. The heart was found beating, and death appeared due to respiratory paralysis.

In rats, the oral lethal dose was 12 Gm./Kg. and the intravenous LD_{50} approximately 3.5 Gm./Kg. Symptoms were similar to those observed in mice, as were the post-mortem findings.

Cats were given three successive daily doses of a 25% solution ranging from 1 to 3 Gm./Kg. All the animals survived, but occasional vomiting occurred after the administration of 2 or 3 Gm./Kg.

PHYSIOLOGICAL EFFECTS

Sucaryl sodium failed to exert any specific action upon the isolated rabbit intestine *in vitro*, or upon the isolated frog heart. The effects observed occurred only with concentrations which resulted in markedly hypertonic solutions and could be duplicated by using an equivalent concentration of an inert salt, as for instance, sodium chloride.

Injections of 100–200 mg./Kg. Sucaryl sodium intravenously in the anesthetized dog did not affect blood pressure and respiration. Similarly, 250 mg./Kg. given within one minute to an anesthetized cat did not change the blood pressure or pulse rate.

In order to investigate any unusual effects upon the central nervous system, one rabbit was given 0.5 cc. of a 1.25% solution and another rabbit was given 0.5 cc. of a 2.5% solution of Sucaryl sodium intracisternally. No effect was observed in the first animal; a transitory convulsion, followed by recovery, occurred in the second.

Nine rats were bilaterally nephrectomized under ether anesthesia. After recovery, two were injected with 2 Gm./Kg.; two others with 4 Gm./Kg. Sucaryl sodium by the intravenous route. The first two animals died three days later; the second two, after one hour. Two of the nephrectomized rats were injected with a dose of sodium sulfate equivalent in sodium content to 4 Gm./Kg. Sucaryl sodium. Both of these animals died within one minute. If sodium sulfate equivalent to 2 or 3 Gm. Sucaryl sodium was injected, death also resulted. This indicates that even if the excretion of the drug by the kidney is prevented, its systemic toxicity can largely be duplicated by using another inert sodium salt.

Of obvious importance was a study of whether this sweetening agent would affect the blood sugar or the metabolism. For this reason, dogs were starved and then injected with 200 mg./Kg. Sucaryl sodium subcutaneously. The blood sugar was followed in one-half, one, two, and three-hour intervals after the injection. No significant changes were observed.

The effect on the basal metabolism rate was studied in rats, using a triple-unit closed-circuit metabolism apparatus designed by Warren E. Collins. An environmental temperature of 28–38°, which has been found by other investigators to be optimal for such work, was maintained. The rats were kept on a diet containing 1% Sucaryl sodium for one month. Basal metabolic rate determinations were conducted prior to their exposure to this diet and at various intervals during the experimental feeding period. The animals were starved for sixteen to eighteen hours prior to each test. After awhile, the rats were placed on a normal diet, and then the procedure was repeated by reverting them to the experimental management. The metabolism of control rats on a standard diet was observed at the same time. The results are shown in Fig. 1. It is evident that Sucaryl sodium did not exert a consistent effect upon oxygen consumption under these conditions. The gradual downward trend with both groups is explained by the increasing ages of the animals.

Possible acute effects upon the oxygen consumption in rats following large single doses were studied on starved animals before and after the intraperitoneal injection of 500 mg./Kg. of this compound. Determinations at one, two, three, and twenty-one hours after the injection failed to show any effect of Sucaryl sodium upon oxygen consumption.

CHRONIC TOXICITY STUDIES

While it thus became obvious that Sucaryl sodium possesses no significant acute toxicity, nor exerts any specific pharmacologic effects, the question of its tolerance by chronic administration remained an important one to answer.

The first studies of this type were directed toward investigating the action of Sucaryl sodium if administered in large repeated doses over a limited period of time. Rats were given 1 Gm./Kg. orally by stomach tube for twenty-one days. No deaths occurred due to the action of the drug, and blood and urine samples taken of individual animals at the end of this treatment showed no pathological changes.

Ten dogs were used for similar purposes. The brief description given of the results obtained in four of them is representative of the group. Two animals were given 2 Gm./Kg. and two others 4 Gm./Kg., daily, for one month. The Sucaryl

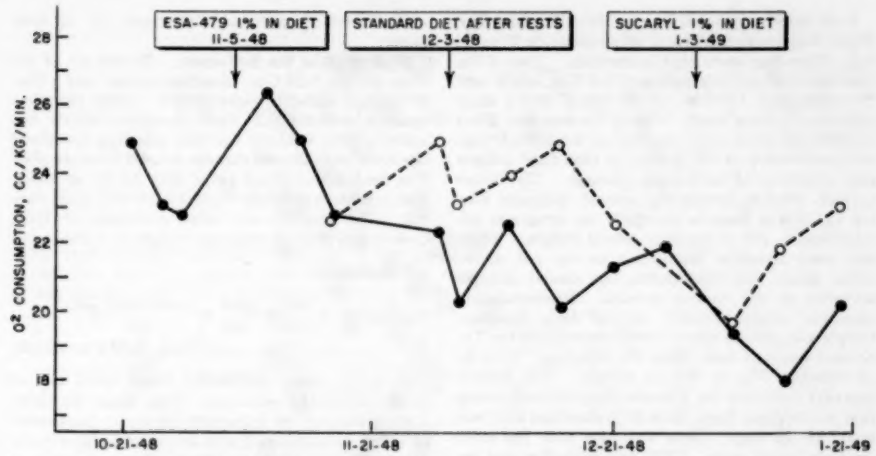


Fig. 1.—Basal metabolic rate of rats receiving Sucaryl sodium. ●—, Sucaryl 1% in breeder's diet—average 4 rats. O– –, breeder's diet controls—average 2 rats.

sodium was first mixed in horse meat but this resulted, sometimes, in vomiting and in the passing of watery stools. Consequently, most of the dogs received the drug by stomach tube. Two of the dogs had gained slightly in weight, one lost some weight, and the other remained unchanged in weight at the end of the experimental period. Urine and blood examinations showed no significant findings and bone marrow punctures performed in two of the dogs revealed a normal picture. Liver function tests were conducted by injecting 5 mg./Kg. bromosulfalein intravenously shortly before the experiment was concluded. In all instances, less than 5% of the dye was found in the plasma one-half hour after the injection, indicating a completely normal performance of the liver in this test.

All animals behaved normally and were in good physical condition when the experiment was concluded. Some of the dogs were killed and autopsied. Histological studies of their inner organs did not show any pathological findings attributable to the treatment. Feeding experiments over long periods of time were conducted in both rats and dogs. Groups of male and female rats, numbering from 8 to 20 animals per group, were placed on a diet containing 0.05, 0.1, or 1.0% Sucaryl sodium, respectively, at the age of twenty-one to twenty-three days, that is, immediately after weaning. The basic diet consisted of a casein-base breeder's diet mixture fortified with all necessary vitamins and accessory foodstuffs. This was changed in some groups during the course of experiments to a diet of powdered commercial rat chow pellets. Some groups of animals were kept for periods of eighteen months, others for twenty-four months, and one group on the 0.05% concentration, for thirty months. Mating was permitted between rats exposed to these diets over a prolonged period of time and the young generation was kept on the same management. It was observed that normal litters were raised by the mother rats and this experiment was conducted into the third generation in one group on a diet of 0.05% of this sweetening agent. Occasionally, animals of the experimental groups died after many months' exposure to this diet, but the death rate was not higher than our regular animal stocks. The incidence of spontaneous tumors, as they are observed in old rats, was likewise no higher than in control animals. During the observation period, repeated complete blood counts were made on representative animals, and the urine pooled from several rats of the same group was examined. No pathological findings were noted. Positive tests for albumen were occasionally found, but these are frequent in old rats. The weight of the experimental male and female rats on the 0.1 and 1% concentration tended to remain somewhat below the normal controls beginning about the eighth week after the exposure to the diet. However, during continuation of the experiments, the average weight of the experimental rats reached, or closely approached, that of the control animals.

At the end of the experimental period, most of the survivors were killed, and their organs studied. Sections of heart, lung, liver, kidney, intestinal tract, spleen, and sex organs did not show findings significantly different from control rats of the same age. In an occasional animal, a few small foci of mononuclear infiltrations in the myocardium were noticed, which led us to conduct a rather extensive study of this observation. For this reason, electrocardiographic recordings were made from a number of rats on this diet and compared with controls of similar age. No significant differences were observed. Further study of this phenomenon indicated that there was no relation between the total dose or concentration administered and the presence or absence of these heart changes which had puzzled us. A review of numerous hearts of control animals showed that similar infiltrations were occasionally present.

It was the opinion of consultant pathologists that these findings could not be attributed to the administration of Sucaryl sodium.

Four dogs were used for chronic administration. Their weights ranged from approximately 8 to 12 Kg. Two dogs were kept as controls. Two of the experimental animals received 0.5 Gm. daily and the other two, 1.0 Gm., orally mixed into a small amount of ground meat. One of the dogs was killed accidentally after three months on test. Histological examination of the tissues at that time did not give evidence of pathologic changes. The other animals showed completely normal behavior during the fifteen months in which the drug was administered. All of the dogs gained weight. There was some irregular fluctuation in the red count, white count, and hemoglobin, but similar changes occurred in the control animals. Bromosulfalein excretion studies showed normal liver function. Only in one animal was a 5–10% retention of the dye present one-half hour after the injection. Usually we consider 5% or less as normal. The kidney function measured by phenolsulfonphthalein excretion was normal (more than 55% excretion after one hour) in all dogs. This was also true for urine analysis, blood sugar, NPN determination and occasional bone marrow smears. Two of the experimental dogs and one control dog were killed at the end of the observation period. Gross autopsy findings were normal and histological sections made of the intestinal tract, bladder, kidney, heart, liver, spleen, and sex organs showed no changes attributable to the treatment of this compound; fat stains of the liver were negative.

EXCRETION STUDIES

Audrieth and Sveda in their original paper (2) reported a study of the fate of this compound in the rabbit. This group found that all material fed to this species could be recovered in the urine. More exactly, 80–90% of this drug was found in the urine twelve hours after intravenous or intraperitoneal injection.

We have conducted corresponding work on the human urinary excretion of Sucaryl sodium. The method used was a modification of that reported by Audrieth and Sveda (2). The phosphates and sulfates were removed from the urine, the sulfamate hydrolyzed with nitrous acid, and the sulfate precipitated with barium chloride and weighed as barium sulfate. A blank on the urine was necessary.

PROCEDURE

Phosphate Removal.—To 100 ml. of urine preserved with toluene and 100 ml. of water, five drops of 1% alcoholic solution of phenolphthalein were added. Concentrated ammonium hydroxide was added dropwise until a pink color persisted. Then 40 ml. of a 5% ammonium chloride solution and 6 Gm. of magnesium carbonate were added. The volume was made to 400 ml., the vessel stoppered and thoroughly shaken for one minute. After settling thirty minutes, the contents were filtered through a dry filter paper into a dry flask.

Ethereal Sulfate Hydrolysis and Sulfate Removal.—To 375 ml. of the above filtrate, 10 ml. of concentrated hydrochloric acid was added and mixed. After standing overnight at room temperature, 3 Gm. of solid barium chloride was added, dissolved, and mixed. After standing one hour, the contents were filtered through dry Whatman No. 42 filter paper.

Hydrolysis of the Sulfamate.—To 360 ml. of the clear filtrate, 0.25 Gm. of sodium nitrite and 1 Gm. of barium chloride were added. After complete solution, another 0.25 Gm. of sodium nitrite was added. The solution was left standing for about one hour, then filtered through a tared filter crucible. The beaker was rinsed twice with 15 ml. of water. The crucible was finally washed with 95% ethyl alcohol. The crucible was dried overnight at 105°, cooled, and dried to constant weight in a desiccator.

Calculation.

$$\text{Mg. } BaSO_4 \times \frac{400}{375} \times \frac{385}{360} \times \frac{\text{total urine vol.}}{100} = \text{mg. } BaSO_4 \text{ in sample.}$$

This value minus the $BaSO_4$ blank gives mg. of $BaSO_4$ due to the sulfamate. This, times the factor 0.86, gives mg. of sulfamate present. Recoveries of 112% were obtained with 14 mg. of sodium cyclohexylsulfamate added to 100 ml. of human urine.

Human Urinary Excretion of Sucaryl Sodium.—Control values were run on the urine of a human subject for six days. On the seventh day, 300 mg. of Sucaryl sodium was taken orally and the urine analyzed daily for twelve more days until the values approached those of the first six days, indicating a return to normal.

In seven days, 79.5% of the dose was found in the urine, with 26% of the total dose being excreted in the first twenty-four hours.

In another experiment in which the same subject ingested 200 mg. Sucaryl sodium, a total of 77% was recovered in three days.

While Sucaryl sodium is a rather inert chemical substance, it seemed of interest to investigate whether or not it would interfere with the usual clinical laboratory tests performed in the routine examination of urine in any way. It was shown that the presence of Sucaryl sodium neither interferes with positive reactions nor gives false positive reactions for the following substances: albumin, glucose, acetone, acetoacetic acid, blood, or the diazo tests.

Effect of Sucaryl Sodium upon Digestive Enzymes.—Since Sucaryl sodium may be used in considerable amounts by persons suffering from various gastrointestinal disturbances, a study of effects in a 1% concentration upon the digestive enzymes (pepsin, trypsin, diastase, and lipase) appeared desirable. The basic procedures followed are standard laboratory methods in general use by enzyme chemists, with certain modifications as noted.

Pepsin.—The procedure employed utilizes the pepsin hydrolysis of a 2% casein solution previously adjusted to a *p*H of 1.5 to 2.2 with HCl (3). The digestion mixture was kept at a temperature of 40° (3), and progress of the digestion determined by conducting formal titrations, using phenolphthalein indicator, on 5-cc. portions withdrawn at the time intervals shown. This procedure was then repeated in exactly the same manner except that 1% of Sucaryl sodium was added to the digestion mixture (see Table I).

Trypsin.—Substantially the same method as used in the pepsin determination was employed, except that the substrate solution was adjusted to a *p*H of

TABLE I.—RESULTS (PEPSIN)

Digestion Time, Min.	Digestion Mixture, Total Titration	Due to Digestion, Ml.	Digestion Mixture Plus 1% Sucaryl Sodium; Total Titration	Due to Digestion, Ml.
0 (blank)	3.05	..	2.88	..
30	3.38	0.33	3.20	0.32
60	3.91	0.86	3.88	1.00
90	3.98	0.93	3.89	1.01

8.0 with barbital sodium buffer and acetic acid, and the digestion mixture was maintained at a temperature of 55° (4). Results are shown in Table II.

TABLE II.—RESULTS (TRYPSIN)

Digestion Time, Min.	Digestion Mixture, Total Titration	Due to Digestion, Ml.	Digestion Mixture Plus 1% Sucaryl Sodium; Total Titration	Due to Digestion, Ml.
0 (blank)	0.18	..	0.10	..
30	0.90	0.72	0.80	0.70
60	1.08	0.90	0.95	0.85
90	1.15	0.97	1.05	0.95

Diastase.—The procedure was based on the assay method for diastase in the U. S. P. IX. However, the preparation of the starch paste was modified as directed in the U. S. P. XIII assay for pancreatin to provide a more uniform substrate. The method is a measurement of the amount of starch digested by the diastase at various time intervals, as indicated by the color produced when two drops of the digestion mixture were added to a dilute iodine solution.

A *p*H of about 7 is considered optimum for the pancreatic diastase digestion (5). The *p*H of the starch-diastase mixture used was found to be 7.10, and the addition of 1% Sucaryl sodium lowered this figure to 7.02.

The digestion was carried out at two levels using 70 mg. and 35 mg., respectively, of diastase U. S. P. IX 1:50, thirty minutes. Results are shown in Table III.

Lipase.—The method used was the improved method of the Willstätter, Waldschmidt-Leitz and Memmen assay as formulated by Bauer and Wilson (6). The method consists of determining the free fatty acids liberated by the hydrolysis of olive oil when pancreatin is added to it. The results are shown in Table IV. From these tests, it is apparent that Sucaryl sodium in a 1% concentration has no appreciable effect on the digestive action of pepsin or trypsin, and slightly enhances the digestive action of diastase and lipase.

TABLE III.—RESULTS (DIASTASE)

Digestion Time, Min.	Color of Iodine Soln. (70 Mg. Diastase)	Color of Iodine Soln. (70 Mg. Diastase + 1% Sucaryl Sodium)
15	Intense blue	Reddish-blue
25	Lighter blue	Reddish-orange
30	Reddish-blue	Light yellow
35	Bluish-red	
40	Reddish	
45	Orange	
50	Yellow	

Digestion Time, Min.	Color of Iodine Soln. (35 Mg. Diastase)	Color of Iodine Soln. (35 Mg. Diastase + 1% Sucaryl Sodium)	Sucaryl Alone
40 Min.	Blue	Blue	Starch paste remained thick throughout period of test, showing no apparent digestion of starch by Sucaryl sodium alone
55	Blue	Blue	
70	Blue	Light blue	
85	Light blue	Violet	
100	Violet	Reddish-orange	

TABLE IV.—RESULTS (LIPASE)

Reaction Mixtures	Titration Values		Blank Titration		Cc. 0.1 *N* KOH Required to Neutralize Liberated Fatty Acids
Pancreatin only	41.55	–	16.40	=	25.15
Pancreatin plus 1% Sucaryl sodium	45.80	–	16.80	=	29.00
Pancreatin plus 1% Saccharin sodium	47.00	–	16.00	=	31.00

DISCUSSION

It appears from the above experiments that Sucaryl sodium has fulfilled the major requirements as a noncaloric dietetic sweetening agent. These are: lack of toxicity both by acute and chronic administration; high sweetening power; pure sweet taste and acceptability by the patient; and pharmaceutical stability. In a recent brief note, Fitzhugh and Nelson (7) are in agreement with our findings regarding the lack of toxicity of Sucaryl sodium following chronic administration in rats. These authors have used as much as 5% of this sweetening agent in the diet without observing anything more than some diarrhea and a slight depression of growth. It will be recalled that diarrhea was also seen by us in experiments with dogs, and this was reported to occur clinically if persons took several grams of Sucaryl sodium at one time for experimental purposes. It appears reasonable to assume that the laxative effect of this material is probably due to an action similar to that of sodium sulfate ingested in larger doses. Otherwise, no specific pharmacologic effects were noticed in the experiments which we have described, other than those which could be explained on the basis of inert salt action.

The excretion of Sucaryl sodium indicates that all, or almost all, of the ingested material appears in the urine in unchanged form. The speed of excretion is not very fast, and this phase is under further investigation in our laboratories. Until more quantitative data are available in this respect, it appears advisable to recommend that Sucaryl sodium should be consumed only with certain precautions by persons suffering from severe renal damage. Our ex-

periment on the nephrectomized rat indicates that Sucaryl sodium behaves about as an equivalent amount of sodium sulfate in such animals.

It is stated (1) that saccharin appears likewise in the urine in unchanged form and is excreted quantitatively within sixteen to forty-eight hours after its administration. Thus, these two sweetening agents show a certain similarity, and the note of caution regarding patients with kidney damage is made only in view of the fact that doses of Sucaryl sodium almost 13 times larger have to be used in order to provide the same sweetening effect as saccharin.

The *in vitro* experiments concerning the action of Sucaryl sodium upon various digestive enzymes make it rather improbable that even large amounts of this sweetening agent would in any way interfere with normal enzymatic digestive processes. If further clinical and experimental evidence confirms and extends our present knowledge of Sucaryl sodium, this sweetening agent should constitute an important and flexible tool in the dietary management of conditions where a potent, noncaloric, safe and stable sweetening material is desired.

SUMMARY

1. Cyclamate sodium (Sucaryl sodium) is a water-soluble, neutral, noncaloric sweetening agent without bitter aftertaste. It is stable in solution and resistant to boiling within a considerable *p*H range.

2. The acute toxicity of Sucaryl sodium is very low; it was found devoid of specific pharmacodynamic effects in the experiments described above.

3. Subchronic and chronic toxicity studies conducted in rats and dogs showed that administration of this sweetening agent over prolonged periods of time and at high dosage levels did not exert any untoward physiological effects, or result in organ changes attributable to the administration of the drug.

4. Sucaryl sodium is nearly quantitatively excreted in the urine in unchanged form.

5. Sucaryl sodium does not depress the activity of digestive enzymes.

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Studies in the Hydrophilic Properties of Possible Ointment Base Constituents. VI. The *n*-Alkyl Amines*

By ALFRED HALPERN,† ROBERT L. MUHICH,‡ and GAETANO SICONOLFI‡

An investigation of the *n*-alkyl amines from C_{10} to C_{18} indicates that these agents are superior emulsifying agents when compared with their analogous alcohol and mercaptan derivatives. The alkalinity of these agents, however, limits their dermatological utility.

IN THE COURSE of a study of the hydrophilic properties of a series of alcohols (1) and mercaptans (2), hydrogen bonding was postulated as a possible explanation for the marked difference in the emulsifying properties of a series of hydroxy and sulfhydryl compounds. The hydrogen-bonding alcohols were far superior as emulsifying agents when compared with their nonassociated mercaptan analogs. It is well known that the amino group is also capable of forming an associated bond, although it is generally conceded to be weaker than that formed by the hydroxy group (3, 4). However, Marvel and co-workers (5), from a study of the solubilities of water and alcohols in amine solvents, concluded that the OH ← N bond is much stronger than the OH ← O bond. Presumably, these types of associated bonds would be present when the amines and alcohols are used as emulsifying agents.

In view of the recent availability of the *n*-alkyl amines,[1] it was deemed of interest to study

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[1] Armour Chemical Division, Chicago, Ill.

these agents and compare their properties with the analogous alcohols and mercaptans, in order to further establish the role of hydrogen bonding in the emulsification process.

EXPERIMENTAL

Commercial samples of the *n*-alkyl amines from C_{10} to C_{18} were obtained from the Armour Chemical Division, Chicago, Ill. These products had a pronounced ammoniacal odor, and also were described as mixtures. The compounds were purified through distillation at 1–2 mm. pressure and a middle cut of the constant boiling fraction was taken.

The *n*-alkyl amines were also prepared through a modification of the well-known Gabriel synthesis. The *n*-alkylphthalimide was obtained by heating under reflux for three to four hours, phthalimide (60 Gm.), anhydrous potassium carbonate (30 Gm.), and the appropriate alkyl halide (2) (20% molar excess). The excess alkyl halide was removed by vacuum distillation and the residue washed with water and dried.

The dried powdered residue was warmed with a 0.1% molar excess of a diluted alcohol solution of hydrazine hydrate. A gelatinous precipitate formed after a few minutes; after sitting at 50–60° for one-half hour, the mixture was made acid with HCl and warmed to 80° for one hour. The insoluble phthalylhydrazide was filtered, and the filtrate concentrated. The cooled solution was made alkaline and extracted with three 25-cc. portions of ether. The ethereal solution was dried over alkali; the solvent was removed and the residue distilled to yield the purified amine. The yields, melting points, and analyses of the test compounds are reported in Table I.

The purified amines, with the exception of *n*-decyl amine (light yellow oil), were white, waxy solids at room temperature. The purified products had a definite melting point and were substantially free from an ammoniacal odor.

The water numbers of the compounds were determined in the usual manner (1) and are reported in Table II. The surface tension of a saturated aqueous solution of the amines and the interfacial tension of an amine-petrolatum-water system were determined with the DuNouy Interface Tensiometer and are reported in Fig. 1 and Table III.

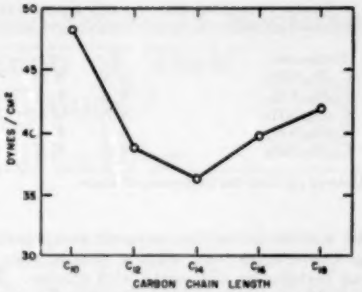

Fig. 1.—Surface tension of a saturated aqueous solution of the *n*-alkyl amines at 25°.

TABLE III.—INTERFACIAL TENSION OF THE *n*-ALKYL AMINE-PETROLATUM WATER SYSTEM AT 55°[a]

Compound	% Concentration of *n*-Alkyl Amine 0.1	0.5	1.0	2.0	3.0
	Dynes/Cm.[b]				
$C_{10}H_{21}NH_2$	28	20	14	8	6
$C_{12}H_{25}NH_2$	24	16	9	5	[b]
$C_{14}H_{29}NH_2$	14	7	3	[b]	[b]
$C_{16}H_{33}NH_2$	10	4	2	[b]	[b]
$C_{18}H_{37}NH_2$	8	2	[b]	[b]	[b]

[a] Determined with the DuNouy Interface Tensiometer, and the usual corrections were applied to the observed data. The reported corrected data represent the average of six observations.
[b] The interfacial tension was too small to measure.

The amine group is known to be fairly strong base. Because of the undesirability of a pronounced alkalinity in dermatological preparations, the *p*H of petrolatum emulsions of the test compounds was studied. A saturated emulsified base containing 5%

TABLE I.—YIELD, MELTING POINTS OF ANALYSES OF THE *n*-ALKYL AMINES

Compound	Yield, %	Melting Point, ° C. Synthetic	Commercial[a]	Recorded[b]	N-Analysis of Synthetic Calcd.	Found[c]
$C_{10}H_{21}NH_2$	73	13–15	11–14	16.1	8.9	8.7
$C_{12}H_{25}NH_2$	68	26–28	24–27	28.3	7.6	7.7
$C_{14}H_{29}NH_2$	76	35–37	32–35	38.1	6.6	6.5
$C_{16}H_{33}NH_2$	74	44–45	40–43	46.7	5.8	5.9
$C_{18}H_{37}NH_2$	78	51–52	48–51	53.0	5.2	5.1

[a] Purified commercial sample.
[b] Ralston, A. W., Hoerr, C. W., Pool, W. D., and Harwood, H. J., *J. Org. Chem.*, **9**, 102(1944).
[c] Average of three determinations.

TABLE II.—THE WATER NUMBERS OF THE *n*-ALKYL AMINES[a]

Compound	% Concentration in White Petrolatum[b] 1	3	5	7.5	10
$C_{10}H_{21}NH_2$	18.6	20.2	24.3	26.4	28.2
$C_{12}H_{25}NH_2$	21.5	24.2	32.1	38.4	43.6
$C_{14}H_{29}NH_2$	26.2	35.7	41.0	47.4	52.1
$C_{16}H_{33}NH_2$	31.2	39.0	46.2	54.1	60.2
$C_{18}H_{37}NH_2$	36.1	48.2	56.1	69.1	78.3

[a] Average of three determinations. The results were checked by the toluene distillation method.
[b] The water number of the petrolatum used was 11.2.

TABLE IV.—THE pH OF A 5% EMULSIFIED PETROLATUM MIXTURE OF THE n-ALKYL AMINES

Compound	Hours pH*								
	0	8	16	24	32	48	72	96	120
$C_{10}H_{21}NH_2$	8.8	8.8	9.0	9.4	9.6	10.0	10.0	10.4	10.8
$C_{12}H_{25}NH_2$	8.6	8.6	9.0	9.2	9.4	9.4	9.6	10.0	10.4
$C_{14}H_{29}NH_2$	8.6	8.8	9.0	9.4	9.6	9.8	10.0	10.2	10.4
$C_{16}H_{33}NH_2$	8.4	8.4	8.6	9.0	9.4	9.8	10.0	10.0	10.4
$C_{18}H_{37}NH_2$	8.2	8.4	8.4	8.8	9.0	9.4	9.8	10.0	10.2

* Determined with the Beckman pH Meter.

of the n-alkyl amine was prepared and stored at room temperature. An aliquot sample was removed periodically and mixed with toluene. The separated aqueous phase was drawn off and the pH determined (Table IV).

The saturated emulsions were rather unstable, and separation was observed in a few days. The mixtures with lesser amounts of water were stable for periods of from four to six weeks. However, in all instances water separated in noticeable drops when the emulsified mixtures were rubbed on the skin. It should be noted, however, that almost all simple petrolatum mixtures of emulsifying agents produced emulsions with similar properties, and that the addition of a stabilizing agent is required in order to produce a satisfactory, stable ointment base. The emulsions were broken by solutions of strong acids, alkali, and electrolytes.

DISCUSSION

The n-alkyl amines, with a carbon length of C_{10} to C_{18} are rather efficient emulsifying agents. The amount of water emulsified by these agents increased with the higher concentration of the emulsifying agent, consequently no optimum concentration was observed. The water numbers of mixture up to 45% were studied, and a slight steady increase of the quantity of water absorbed was noted. These higher concentrations produced mixtures which did not have the properties of an ointment, and were thick viscous fluids.

The role of hydrogen bonding in emulsion formation is again brought forth since the OH←N bond is reportedly (5) stronger than the OH←O bond. This may account for the superior emulsifying properties of n-alkyl amines when compared to the analogous alcohols. A drawback of these agents is their inherent alkalinity, which excludes this class of compounds from general dermatological practice. A limited patch testing of the 5% emulsions of the test agents produced a marked erythema and irritation in all instances.

While it is not conclusively established that alkalinity is the sole source of irritability of these agents, it nevertheless is a major contributing factor.

SUMMARY AND CONCLUSIONS

1. The n-alkyl amines from C_{10} to C_{18} were prepared, and their physical properties noted.

2. The pharmaceutical utility of these compounds was investigated and the water numbers, surface tensions, and interfacial tensions are reported.

3. The n-alkyl amines have a distinct alkalinity which limits their dermatological use.

4. The n-alkyl amines studied were superior in emulsifying ability to the analogous alcohols and mercaptans.

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The Preparation of Five Cation-Saturated Bentonites and the Determination of Their Exchangeable Cations*,†

By MARTIN BARR‡ and EARL P. GUTH§

Procedures are described for the preparation of five cation-saturated bentonites. The principle employed involves the phenomenon of cation exchange in clays. The determinations of the exchangeable cations in the bentonites were carried out using a modified procedure of Mehlich's original method for the same determination in soils.

THE CLAYS which are referred to as bentonites may be divided into two general classes: the sodium bentonites, which absorb large quantities of water, swelling enormously in the process and having the property of remaining in thin water dispersions; and the calcium bentonites, which absorb very little water, do not swell noticeably, and settle rapidly in thin water dispersions (1). The Black Hills region of Wyoming and South Dakota supply the greatest quantity of sodium bentonite (2), and the calcium bentonite, although widespread in occurrence, is produced mostly in Monroe County, Mississippi.

In considering bentonite, it must be borne in mind that this substance belongs to a large group of clay materials. Most clays are composed essentially of minute particles of one or more of the clay minerals, of which the kaolinite, montmorilonite, and illite groups are the most important. The clay minerals occur in flake-shaped particles, possess a base-exchange capacity, and exist in, or are reducible to, extremely small grain sizes on working with water. Different clay minerals possess these properties in varying degrees. The clay minerals, being the primary constituents of clay materials, are the most important single factor in determining the properties of these materials (2).

Bentonite belongs to the montmorilonite group of clay minerals, so named because it takes its name from the mineral montmorilonite, having the composition $(OH)_4Al_4Si_8O_{20} \cdot H_2O$. The aluminum is usually partly replaced by magnesium and iron. Montmorilonite constitutes 90 per cent of the various bentonites. The other 10 per cent consists of minute fragments of other minerals, the most abundant being feldspar (3). The average chemical analyses of two brands of bentonite obtainable from the American Colloid Company are listed below (1):

AVERAGE CHEMICAL ANALYSES

(Moisture Free)

	Volclay Bentonite, %	Panther Creek Bentonite, %
Silica	64.00	64.00
Alumina	21.00	17.10
Iron	3.50	4.70
Magnesia	2.30	3.80
Lime	0.50	1.50
Soda	2.60	0.20
Potash	0.40	0.50
Minor	0.50	0.20
Chemical Water	5.20	8.00

Base Exchange in Clays.—Clay materials have the property of carrying cations that may be exchanged stoichiometrically for other cations by treatment with a water solution containing the second cation (4). This process of cationic exchange, or base exchange, as it is generally called, does not alter the structure of the material, but it is a very important factor influencing the properties of clays and soils.

Thompson (5) and Way (6–8) were the first to initiate the modern study of this subject, both being primarily concerned with the possible loss of soluble fertilizers from soils by leaching. Way had no difficulty in proving that the active ingredient of the soil, in absorption of bases, was its clay, and that the constituent necessary for this exchange to take place was hydrated aluminosilicates of alkali or alkaline earths. Ross and Shannon (9) in 1926 had indicated the presence of montmorilonite, the chief constituent of bentonite in soils, and by 1930 it became generally recognized that montmorilonite was a common constituent of many soils. Kelley, Dore, and Brown (10), studying the determination of base-exchange reactions of bentonites, found that the exchangeable cations vary with the source of the bentonite. They state that since X-ray analysis indicates an orderly arrangement of the atoms

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within the particles, and since the exchange of bases is stoichiometric, it is safe to conclude that the base-exchange substances are true chemical compounds. The replaceable bases, therefore, are not merely adsorbed on the surface of the particles as Wiegner contended (11), but rather the bases are an integral part of the chemical constitution of the crystals.

Concerning the rate of cation exchange, Gedroiz (12) and Hissink (13) have presented data which substantiate Way's conclusion that the exchange reaction takes place practically as soon as contact is established between the clay and solution. The temperature coefficient of cation exchange is generally small (14). Wiegner (15) found a small negative temperature coefficient, while other workers (16–17) have found that the exchange reaction is accelerated somewhat by raising the temperature. However, the disadvantage of increasing the temperature may outweigh the advantage, as Chapman and Kelley (18) have shown that heating above room temperature may increase the solubility of certain constituents and thus magnify the error which is always involved to some extent in cation-exchange determinations.

Among others, Schachtschabel (19) has investigated the influence of the effect of concentration of solutions on the cation exchange of clay material. His results demonstrated quite conclusively that the effects of concentration depend on what kind of cation is being replaced, and also on the nature of the replacing ion. With cation pairs of similar replacing power and of the same valence such as K^+ *vs.* NH_4^+ or Ca^{++} *vs.* Ba^{++}, dilution has very little effect on exchange, while with cations of different replacing power and different valence, for example, Na^+ *vs.* Ca^{++}, or NH_4^+ *vs.* Ca^{++}, dilution produces marked effect on exchange. Many investigators have shown that the replacing power of different kinds of cations differ substantially. If equal amounts of a clay that is saturated with some base, barium for example, are treated with any given concentration of a chloride solution of either sodium, potassium, magnesium, calcium, or hydrogen, amounts of barium which have become replaced are found to stand in the order: $Na > K > Mg > Ca > H$, i.e., Na has less replacing power than K, K less than Mg, Mg less than Ca, Ca less than H (20). Vanselow (21) showed that part of this difference is caused by differences in the activities of these ions. Wiegner (22) holds that differences in degree of hydration of these ions is primarily responsible for the results.

In order subsequently to study the properties of bentonites saturated with various cations, advantage was taken of the cation-exchange properties of clays to prepare five different cation-saturated bentonites from Volclay bentonite.[1] It is with the methods of manufacture of these bentonites and the determination of their exchangeable cations that this paper is concerned.

EXPERIMENTAL

Preparation of Bentonites

Sodium bentonite, potassium bentonite, calcium bentonite, magnesium bentonite, and hydrogen bentonite were prepared from Volclay bentonite. They will be referred to throughout this paper as Na bentonite, K bentonite, Ca bentonite, Mg bentonite, and H bentonite, respectively. When Na bentonite is referred to, it is to imply that the exchangeable cations of that bentonite consist of sodium, if not completely so, nearly completely so. The same is meant when referring to the other bentonites, except that their exchangeable cations consist of the cations referred to in their respective titles. The methods of preparation of each bentonite are principally similar. A complete method for the preparation of Na bentonite will be given. The methods for the preparations of the other bentonites will be given, listing procedure only where the manufacture differs from that involved in preparing Na bentonite.

Preparation of Na Bentonite.—Twenty grams of Volclay bentonite and 175 ml. of 1 *M* sodium acetate solution were shaken in a 250-ml. centrifuge bottle for thirty minutes in a mechanical shaker.[2] The mixture was then centrifuged,[3] and the supernatant discarded. The centrifuged bentonite was then shaken for ten minutes with another 175 ml. of 1 *M* sodium acetate solution, again centrifuging and discarding the supernatant. This procedure was repeated four times in order to insure complete replacement of a maximum of the exchangeable cations in bentonite for sodium. The centrifuged Na bentonite was then washed free of excess sodium acetate by shaking once with 175 ml. of 50% w/v alcohol for ten minutes, centrifuging and discarding the supernatant, and then shaking five times with 175-ml. portions of 80% w/v alcohol for ten minutes, again centrifuging and discarding the supernatant. The bentonite was shaken twice with 100 ml. of 95% w/v alcohol for ten minutes to rid it of excess water, centrifuged, and the supernatant discarded. The bentonite was shaken with variable portions of 95% w/v alcohol and transferred to a six-inch evaporating dish. The alcohol was allowed to evaporate spontaneously, and the bentonite was then dried on a hot plate at 110° for twenty-four hours. The Na bentonite was then reduced to a fine powder by processing it in a micro-pulverizer.

Preparation of K Bentonite.—The preparation of K bentonite differs from that of Na bentonite in the following respects: (*a*) 1 *M* potassium acetate solution replaces 1 *M* sodium acetate solution as the leaching solution, and (*b*) the centrifuged bentonite is washed free of excess potassium acetate as follows: it is twice shaken with 175-ml. portions of distilled

[1] B. C. Volclay Bentonite, American Colloid Co.
[2] Precision Scientific Co., Cat. No. 5855.
[3] Size 2 Centrifuge of International Equipment Co.

water for ten minutes, centrifuged, and the supernatant discarded. It is then shaken three times with 175-ml. portions of 80% w/v alcohol for ten minutes, centrifuged, and the supernatant decanted. Finally, it is shaken twice for ten minutes with 95% w/v alcohol, centrifuged, and the supernatant decanted.

Preparation of H Bentonite.—The preparation of H bentonite differs from that of Na bentonite in the following respects: (*a*) 1 *M* acetic acid replaces 1 *M* sodium acetate as the leaching solution, and (*b*) the centrifuged bentonite is washed free of excess acetic acid in a manner similar to that in which excess potassium acetate is washed from K bentonite.

Preparation of Ca Bentonite.—The preparation of Ca bentonite differs from that of Na bentonite in the following respects: (*a*) 1 *M* calcium acetate replaces 1 *M* sodium acetate as the leaching solution, and (*b*) the excess calcium acetate is washed free from the centrifuged bentonite as follows: it is shaken five times with 175-ml. portions of distilled water for ten minutes, centrifuged, and the supernatant decanted. It is then washed three times with 175-ml. portions of 95% w/v alcohol for ten minutes, centrifuged, and the supernatant discarded.

Preparation of Mg Bentonite.—The preparation of Mg bentonite is similar to that described for the preparation of Ca bentonite except that 1 *M* magnesium acetate solution is used as the leaching solution instead of 1 *M* calcium acetate.

Determination of the Exchangeable Cations of Na, K, H, Ca, Mg, and Volclay Bentonites

The exchangeable cations of Na, K, H, Ca, Mg, and Volclay bentonites were determined by a modified procedure of an original method reported by Mehlich (23) for the determination of exchangeable cations in soils. The modification was necessary in view of the difference of the physical properties of the bentonites as compared to soils chiefly swelling. The modification, which was necessary, involved the procedure for the extraction of the exchangeable cations from the bentonite. The procedure used for the quantitative determination of the individual cations, once they had been extracted, is that exactly as described by Mehlich. The modification employed is as follows:

"Put 2.5 Gm. of bentonite (air-dried and screened through a 1-mm. sieve) into a 50-ml. centrifuge tube. Add 50 ml. of replacement solution *A*,[4] shake in a mechanical stirrer for thirty minutes, centrifuge, and filter the supernatant into a 250-ml. volumetric flask. Repeat this procedure with another 50-ml. portion of replacement solution *A*. After this, add 25 ml. of replacement solution *B*[5] to the centrifuged bentonite, shake for thirty minutes, centrifuge, and filter into a 250-ml. volumetric flask. Repeat this procedure three times using 40-ml. quantities of distilled water, and add distilled water until the extracting solution has the volume of 250 ml. Aliquot portions of this extract are then used for determining the exchangeable hydrogen, calcium, magnesium, potassium, and sodium."

Exchangeable hydrogen is determined in this procedure by titrating an aliquot of the extract with 0.04 *N* HCl to a pink color with a special mixed indicator.[6] The hydrogen, in milliequivalents, in the bentonite is the difference between the titration of the unknown and the titration of an equal aliquot of replacement solution *A*.

Exchangeable calcium is determined by precipitating the cation from an aliquot of the extract as calcium oxalate, forming oxalic acid by adding hot sulfuric acid, and titrating the oxalic acid with standard $KMnO_4$. The milliequivalents of calcium in 100 Gm. of bentonite may be obtained from the general formula stated below.

Exchangeable magnesium, potassium, and sodium are determined colorimetrically. Magnesium is determined by forming a color complex with thiazole yellow after the calcium has been removed from an aliquot of the extract. Potassium is determined by precipitating the cation as potassium cobaltinitrite, dissolving in concentrated sulfuric acid, and forming a color complex with sodium-acetate-nitroso-R-salt solution. Sodium is determined by precipitating as sodium uranyl magnesium acetate, dissolving the precipitate in water, and forming a color complex with 0.35 *N* sulfosalicylic acid. All color complexes were measured using the Fischer Electrophotometer, A.C. Model. The wave lengths of light used for the individual colorimctric determinations are listed in Mehlich's original procedure.

The milliequivalents of the above cations in 100 Gm. of bentonite may be calculated using the following general formula:

$$\frac{\text{Mg. exchangeable cation in 100 Gm.} \times \text{valence of cation}}{\text{Mol. wt. cation}} = \text{Milliequivalents of cation per 100 Gm. of bentonite}$$

The exchangeable metallic bases in milliequivalents per 100 Gm. of the individual bentonites are listed in Table I.

DISCUSSION

The experimental results recorded in Table I indicate that it was possible to take advantage of the cation exchange phenomenon in clays to produce bentonites saturated with different cations. The bentonites prepared were actually not completely saturated with their respective cations, but were to a sufficient degree to make it possible in future studies to observe the effects of the cations on the bentonite structures.

The differences in the preparation of the various bentonites consisted essentially in the manner in which the excess leaching salt was washed from the saturated bentonites. This was necessary because of the effects of the different cations on the hydration of the bentonites. For instance, it was observed that Na bentonite was the most hydrating of the bentonites, followed by K, Volclay, H, Ca, and Mg bentonites in descending hydrating order. Therefore, it

[4] Replacement solution *A*, barium chloride-triethanolamine: Dilute 90 ml. of commercial triethanolamine (sp. gr. 1.126) with 1,000 ml. of water and partly neutralize with HCl to *p*H 8.1. Make up to 2 L. with water and mix with 2 L. of a solution containing 100 Gm. $BaCl_2 \cdot H_2O$. Protect from CO_2 of air during storage.

[5] Replacement solution *B*, barium chloride: Dissolve 25 Gm. $BaCl_2 \cdot 2H_2O$ in 1 L. of water.

[6] Mixed indicator: Triturate 0.1 Gm. bromocresol green indicator with 14.3 ml. of 0.01 *N* NaOH and dilute to 200 ml. with water. Dissolve 0.1 Gm. of methyl red in 200 ml. of 95% alcohol. Mix the indicators to consist of 2 parts bromocresol green and 2 parts methyl red.

TABLE I.—EXCHANGEABLE METALLIC BASES IN MILLIEQUIVALENTS PER 100 GM. BENTONITE

	Volclay	K	Na	Ca	Mg	H
Sodium	83.2	4.4	97.0	4.0	3.3	3.6
Potassium	4.7	99.6	2.6	2.0	1.2	1.1
Calcium	20.9	4.3	7.3	95.1	4.2	2.2
Magnesium	1.2	0.3	0.6	0.9	93.6	0.3
Hydrogen	2.0	1.1	1.1	1.1	1.0	104.3

was not possible to use distilled water indiscriminately in washing the excess leaching salts out from the Na, K, and H bentonites, as their hydration and swelling would not permit successful removal of the salts. With the Ca and Mg bentonites, which do not hydrate to any appreciable degree, water could be used without caution. It was necessary to adjust the washing procedures for the more hydrating bentonites to produce effective manufacture by using 80% w/v alcohol as the washing solution to prevent appreciable interfering swelling.

There are various methods available for the determination of exchangeable cations in soils and clays. Among others may be mentioned those described by Bower and Troug (24, 25), Ross and Hendricks (26), and Mehlich (23). The quantitative determination of exchangeable cations depends principally on the complete replacement of all exchangeable cations by some cation which is not present in the sample, and then determining the amount of cation going into solution from the clay material or by determining the loss of cations from the clay material. Analysis of the leachate is obviously more simple, and is used the most often. Most of this work has been done on soils and modifications are necessary to use these methods for the determination on the bentonites, especially the swelling types.

Any salt of an appreciably potent replacing cation may be used in the leaching solution, but usually a worker develops his own desirable choice. Neutral normal ammonium acetate solution is especially well adapted to this determination and is widely used. It was decided in this study to use Mehlich's method for the determination of exchangeable cations. This process was devised for soils, but because of the swelling properties of bentonite, it was not possible to follow his exact procedure, hence the stated modifications were necessary.

SUMMARY

1. Five different cation-saturated bentonites were prepared by essentially the same method from Volclay bentonite by utilizing the principle of cation exchange in clays.

2. The method described by Mehlich for the determination of exchangeable cations in soils was modified so that it could be applied to the same determination in the bentonites.

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Cation-Saturated Bentonites as Constituents of Ointment Bases*,†

By MARTIN BARR‡ and EARL P. GUTH§

Ointment bases have been prepared utilizing Na, K, Ca, Mg, H, and Volclay bentonites as major constituents of these bases. Observations are reported on the relative swelling power of these bentonites, and the color intensity and *p*H of their bases. Anti-infective ointments were prepared utilizing these bases as carriers for the drugs, and studies of the bacteriological activity of these ointments are reported. These ointments show greater antibacterial activity than their respective U. S. P. and N. F. ointments. Sulfathiazole, phenol, and ammoniated mercury showed greater activity when incorporated into a H bentonite base than when these drugs were incorporated into the other bentonite bases.

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BENTONITE has frequently been mentioned in the literature (1–8) as an ingredient in ointment bases. Darlington and Guth (9) recently reported some phases of their investigation of bentonite as a major component of ointment bases. The bentonite used in these studies was Volclay bentonite,[1] a montmorilonite clay containing exchangeable cations of sodium, calcium, potassium, magnesium, and hydrogen—sodium predominating.

By taking advantage of the cation exchange properties of clays, the authors (10) recently reported the preparation of five cation-saturated bentonites: Na, K, Ca, Mg, and H bentonites. Ointment bases were prepared using these bentonites as major constituents of the bases. Anti-infective ointments were then prepared using these various bentonite bases as vehicles for the anti-infective drugs. Studies were then made on these preparations to ascertain any effect that the different cations of the bentonites might have on the properties and bacteriological activity of the ointments. It is with these studies that this paper is concerned.

EXPERIMENTAL

Part I—Formulation of Bentonite Bases and Ointments

Bentonite bases were prepared containing Volclay bentonite and the cation-saturated bentonites as major components. Volclay bentonite base was prepared according to the formula of Darlington and Guth (9). The other bases were formulated using different percentages of the individual bentonites than was the case with Volclay bentonite, due to a difference in swelling produced by these various cation-saturated bentonites. The bentonite bases were prepared by adding the individual bentonites to the liquid portions of the bases in a mechanical mixer at a slow[2] rate of speed, and stirred until a homogeneous preparation resulted. The formulas of the bases are as follows:

BASE 1

Volclay Bentonite Base

	w/w, %
Volclay Bentonite	20.0
Glycerin	10.0
Distilled Water	70.0

BASE 2

Na Bentonite Base

	w/w, %
Na Bentonite	13.0
Glycerin	10.0
Distilled Water	77.0

BASE 3

K Bentonite Base

	w/w, %
K Bentonite	19.0
Glycerin	10.0
Distilled Water	71.0

BASE 4

H Bentonite Base

	w/w, %
H Bentonite	40.0
Glycerin	10.0
Distilled Water	50.0

BASE 5

Ca Bentonite Base

	w/w, %
Ca Bentonite	38.0
Glycerin	10.0
Distilled Water	52.0

BASE 6

Mg Bentonite Base

	w/w, %
Mg Bentonite	35.0
Glycerin	10.0
Distilled Water	55.0

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[1] B. C. Volclay Micron Bentonite, American Colloid Co.

[2] Number one speed, Hamilton Beach Mixer, Model E.

Ointments of sulfathiazole (5% w/w), ammoniated mercury (5% w/w), boric acid (10% w/w), phenol (2% w/w), and iodine (7% w/w) were incorporated into each of the prepared bentonite bases using proper pharmaceutical technique. Pharmaceutical necessities such as potassium iodide and iodine were used in the same concentrations as are found in iodine ointment, N. F. VIII, and phenol ointment, U. S. P. XIII.

*p*H Determinations of the bases and ointments were made and recorded in Table I.

Discussion.—It was found that the exchangeable cation content of the bentonites influences its degree of swelling in the order of Na > K > Mg > Ca > H. Volclay bentonite was found to swell to a greater degree than Mg bentonite and less than K bentonite. This order of the effect of exchangeable cations on the swelling of bentonite is in agreement with the work of Baver and Winterkorn (11). Therefore, due to this varied swelling, it was necessary to use various percentages of the bentonites in the preparation of their respective bases in order to prepare bases of suitable ointment-like consistency.

The different bentonites were also found to produce ointments of slightly different color. All the bases were of a brownish-tan color, and the cations had an influence on the intensity of the color in the order Na > K > Mg > Ca > H. Volclay bentonite produced a base having a color intensity between that of K and Mg bentonites.

Also, the different bentonites were found to influence the *p*H of the bases in the following order: Na > K > Mg > Ca > H—Na bentonite being the most alkaline. Volclay bentonite produced a base having an alkalinity between that of Ca and Mg bentonites.

It is worthy of note that the order of the effect of the different bentonites on the swelling, color, and *p*H of the bases is similar. The property of the antiinfectives incorporated into the bases was apparently more significant in the determination of the *p*H of the resulting ointment than was the *p*H of the base from which the ointment was prepared.

It was observed that the addition of boric acid to the various bentonite bases reduced the consistency of the Na, K, and Volclay bentonite bases. This is probably due to the hydrogen ions of the boric acid exchanging positions with the cation of the prepared bentonites. Hence, as previously, the hydrogen ion produces less swelling effect on the bentonites than do the other more hydrating sodium and potassium cations. Therefore, the bentonite bases of Na, K, and Volclay bentonite lost some of their consistency. When added to the H, Ca, and Mg bentonite bases, the hydrogen cation of the boric acid does not produce this effect, for although the cation exchange is still occurring, the swelling effects of hydrogen, calcium, and magnesium cations on the bentonites are similar enough that the change in consistency of the bases is not noticeable. Indeed the boric acid, 10% w/w, increases the consistency of these bases somewhat by mere addition of bulk. Ammoniated mercury and sulfathiazole, when added to each of the bases, increased the consistency of these bases. This also may be attributed to addition of bulk to the bases. The incorporation of phenol or iodine to the bases did not produce a visible change in consistency.

It was necessary to add preservatives to the bentonite bases and the ointments containing sulfathiazole to prevent mold growth. As reported by Darlington and Guth (9), methylparaben 0.15% and propylparaben 0.05% were used as the preservatives.

Part II—Cup-Plate Tests of Bases and Ointments

The bases and ointments prepared in Part I were tested for their bacteriological activity using the F.D.A. cup-plate method (12). The organisms

TABLE I.—RESULTS OF *p*H DETERMINATIONS OF BENTONITE BASES AND OINTMENTS

5% Sulfathiazole in Base	*p*H of Base	*p*H of Ointment	*p*H Difference	5% Ammoniated Mercury in Base	*p*H of Base	*p*H of Ointment	Differ
1	8.29	7.70	−0.59	1	8.29	8.11	−0.18
2	8.80	7.77	−1.03	2	8.80	8.50	−0.30
3	8.55	7.76	−0.79	3	8.55	8.30	−0.25
4	3.70	3.82	+0.12	4	3.70	4.75	+1.05
5	8.00	7.58	−0.42	5	8.00	7.89	−0.11
6	8.59	7.97	−0.62	6	8.59	8.45	−0.14
10% Boric Acid in Base				2% Phenol in Base			
1	8.29	5.37	−2.92	1	8.29	7.37	−0.92
2	8.80	5.20	−3.60	2	8.80	7.50	−1.30
3	8.55	5.10	−3.45	3	8.55	7.90	−0.65
4	3.70	3.32	−0.42	4	3.70	4.50	+0.80
5	8.00	5.58	−2.42	5	8.00	7.60	−0.40
6	8.59	5.40	−3.19	6	8.59	7.85	−0.74
7% Iodine in Base							
1	8.29	5.30	−2.99				
2	8.80	5.35	−2.45				
3	8.55	5.40	−3.15				
4	3.70	4.00	+0.30				
5	8.00	5.15	−2.85				
6	8.59	5.25	−3.34				

against which the bases and ointments were tested were *S. typhosa* (U.S.D.A. strain), *S. aureus* (U.S.-D.A. 209 strain), and *E. coli* (Levine strain). The stock cultures[1] were kept on beef agar slants at 4° and subcultured every thirty days. Ammoniated mercury ointment (U. S. P. XIII), boric acid ointment (U. S. P. XIII), phenol ointment (U. S. P. XIII), iodine ointment (N. F. VIII), sulfathiazole 5% in white ointment (U. S. P. XIII), and white ointment (U. S. P. XIII) were included in the testing procedure.

[1] Supplied by Department of Bacteriology, The Ohio State University.

The results of the bacteriological tests of the ointments and bases are listed in Table II.

Discussion.—The general order of effectiveness of the anti-infectives tested in the various bentonite bases as evidenced by comparison of the produced zones of inhibition of growth recorded in Table II was: against *S. aureus* and *S. typhosa*, iodine, boric acid, ammoniated mercury, phenol, and sulfathiazole; and against *E. coli*, iodine, boric acid, phenol, ammoniated mercury, and sulfathiazole.

TABLE II.—RESULTS OF BACTERIOLOGICAL TESTS ON BENTONITE BASES AND OINTMENTS

5% Sulfathiazole in Base	*S. aureus* Zone of Inhibition, Mm.	*E. coli* Zone of Inhibition, Mm.	*S. typhosa* Zone of Inhibition, Mm.
White Ointment	0.0	0.0	0.0
1	0.0 (3.0)[a]	0.0 (13.0)[a,b]	0.0 (3.0)[a,c]
2	0.0 (3.0)[a]	0.0 (13.0)[a,b]	0.0 (3.0)[a,c]
3	0.0 (3.0)[a]	0.0 (13.0)[a,b]	0.0 (3.0)[a,c]
4	1.0 (3.0)[a]	0.0 (15.0)[a,b]	5.0[c]
5	0.0 (3.0)[a]	0.0 (13.0)[a,b]	0.0 (3.0)[a,c]
6	0.0 (3.0)[a]	0.0 (13.0)[a,b]	0.0 (3.0)[a,c]
5% Ammoniated Mercury in Base			
U. S. P. XIII	4.0	1.0	3.0
1	7.0	4.5[b]	7.0[c]
2	7.0	4.5[b]	7.0[c]
3	7.0	4.5[b]	7.0[c]
4	11.0	9.0[b]	10.5[c]
5	7.0	4.5[b]	7.0[c]
6	7.0	4.5[b]	7.0[c]
10% Boric Acid in Base			
U. S. P. XIII	0.0	0.0	0.0
1	13.5	6.5[b]	11.5[c]
2	13.5	6.5[b]	11.5[c]
3	13.5	6.5[b]	11.5[c]
4	13.5	6.5[b]	11.5[c]
5	13.5	6.5[b]	11.5[c]
6	13.5	6.5[b]	11.5[c]
2% Phenol in Base			
U. S. P. XIII	0.0	0.0	0.0
1	6.5	6.5[b]	7.5[c]
2	6.5	6.5[b]	7.5[c]
3	6.5	6.5[b]	7.5[c]
4	7.5	7.5[b]	8.5[c]
5	6.5	6.5[b]	7.5[c]
6	6.5	6.5[b]	7.5[c]
4% Iodine, 4% KI in Base[d]			
N. F. VIII	29.0	1.0	15.0
1	Complete	Complete	Complete
2	Complete	Complete	Complete
3	Complete	Complete	Complete
4	Complete	Complete	Complete
5	Complete	Complete	Complete
6	Complete	Complete	Complete
Bentonite Bases			
White Ointment	0.0	0.0	0.0
1	0.0	0.0[b]	0.0[c]
2	0.0	0.0[b]	0.0[c]
3	0.0	0.0[b]	0.0[c]
4	0.0 (2.0)[a]	3.0[b]	0.0 (4.0)[a,c]
5	0.0	0.0[b]	0.0[c]
6	0.0	0.0[b]	0.0[c]

[a] Zone of partial inhibition of growth.
[b] Zone of apparent stimulation of growth.
[c] Zone of apparent slight stimulation of growth.
[d] Plates incubated at room temperature.

The results of the bacteriological tests clearly indicated that the tested anti-infectives had greater antibacterial activity when incorporated into the various bentonite bases than when incorporated into fatty bases. Only ammoniated mercury and iodine were observed to have any antibacterial properties when incorporated into a fatty base. Phenol, sulfathiazole, and boric acid, while showing no activity in the fatty bases, did show antibacterial properties when applied in the bentonite bases.

The iodine ointments, incorporated into the bentonite bases, gave complete zones of inhibition throughout the test plates, when incubated at room temperature or in an incubator. It was found on assay that approximately 80% of the iodine diffused from its bentonite bases into the test media.

It is shown in Table II that the effect of anti-infectives on the test organisms when incorporated into the bentonite bases saturated with sodium, potassium, calcium, and magnesium, gave similar results as the Volclay bentonite. Therefore, it may be concluded that the Na, K, Ca, and Mg bentonites have no advantage over Volclay bentonite.

However, there was some difference in bacteriological activity produced when H bentonite base was used as the carrier. Sulfathiazole, ammoniated mercury, and phenol showed a greater antibacterial action against the test organisms when these anti-infectives were incorporated into H bentonite base rather than into the other alkaline bentonite bases. In the control tests, the H bentonite base alone also exhibited a greater antibacterial activity against the test organisms than the alkaline bentonite bases. It is noted also that the *p*H of the ointments of ammoniated mercury, sulfathiazole, and phenol in H bentonite base have an acid *p*H, whereas the ointments of the same drugs in the alkaline bentonite bases have an alkaline *p*H. It is also recognized that the boric acid ointments demonstrated the same antibacterial activity regardless of the bentonite base in which it was incorporated. The *p*H of all the boric acid ointments was on the acid side, regardless of the bentonite bases used. This would seem to indicate that the *p*H of the anti-infective ointments is a significant factor in the antibacterial activity of the ointments. Where the *p*H of the ointment of an anti-infective drug (ammoniated mercury, sulfathiazole, phenol) was on the acid side, there was greater bactericidal activity than when ointments of the same drugs had an alkaline *p*H. Therefore, it may be stated that bentonite, when saturated with hydrogen cation and used as a base for ammoniated mercury, sulfathiazole, and phenol ointment has an advantage over the alkaline bentonites.

Darlington and Guth (9) have reported the production of zones of apparent stimulation of growth when testing the bentonite bases and ointments against *E. coli* and *S. typhosa*. The same phenomenon was exhibited in this study. Investigation is being continued in an effort to explain this phenomenon.

SUMMARY

1. Bentonite bases having satisfactory ointment-like consistencies may be prepared utilizing the various cation-saturated bentonites as the major constitutents.

2. Sulfathiazole, ammoniated mercury, boric acid, phenol, and iodine may be satisfactorily incorporated into these bases.

3. The cation content of the bentonites influences the swelling degree, the intensity of the brownish-tan color and the *p*H of the bentonite bases.

4. Bentonite bases are superior to the U. S. P. and N. F. bases as carriers for the anti-infective drugs tested, judging from results obtained utilizing the F.D.A. cup-plate method of assay for antiseptic ointments.

5. Na, K, Ca, and Mg bentonites have no advantages as ointment base constituents over Volclay bentonite for the anti-infectives tested in the cup-plate method.

6. Sulfathiazole, ammoniated mercury, and phenol show greater antibacterial activity against the test organisms when incorporated into H bentonite base than when incorporated into Volclay, K, Na, Mg, and Ca bentonite bases.

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A Study of the Formation of Zones of *p*H Change and Zones of Apparent Stimulation of Growth by *E. coli* and *S. typhosa* upon Bacteriological Testing of Some Glycerin-Containing Ointments*,†

By MARTIN BARR‡ and EARL P. GUTH§

IN A PREVIOUS paper (1), results of bacteriological tests of anti-infective ointments utilizing bentonite bases and of bentonite bases themselves were reported. The F. D. A. cup-plate method of determination was used in these tests. The appearance of zones of apparent stimulation of growth, which were produced when *Escherichia coli* and *S. typhosa* were the test organisms, was reported.

In an effort to study this phenomenon, the bacteriological testing of these ointments, and other control ointments, was repeated, utilizing a modified F. D. A. cup-plate method, making use of bromocresol purple indicator. It is with this study that this paper is concerned.

EXPERIMENTAL

Two sets of bentonite bases were prepared, bases 1 to 6 and bases 1A to 6A. The two series differed in that bases 1 to 6 contained glycerin and bases 1A to 6A did not. Bases 1 to 6 are the same as reported previously (1). The bases and their general method of preparation are listed below:

Base 1	w/w, %	*Base 1A*	w/w, %
Volclay Bentonite	20.0	Volclay Bentonite	20.0
Glycerin	10.0	Distilled Water	80.0
Distilled Water	70.0		

Base 2	w/w, %	*Base 2A*	w/w, %
Na Bentonite	13.0	Na Bentonite	13.0
Glycerin	10.0	Distilled Water	87.0
Distilled Water	77.0		

Base 3	w/w, %	*Base 3A*	w/w, %
K Bentonite	19.0	K Bentonite	19.0
Glycerin	10.0	Distilled Water	81.0
Distilled Water	71.0		

Base 4	w/w, %	*Base 4A*	w/w, %
H Bentonite	40.0	H Bentonite	40.0
Glycerin	10.0	Distilled Water	60.0
Distilled Water	50.0		

Base 5	w/w, %	*Base 5A*	w/w, %
Ca Bentonite	38.0	Ca Bentonite	38.0
Glycerin	10.0	Distilled Water	62.0
Distilled Water	52.0		

Base 6	w/w, %	*Base 6A*	w/w, %
Mg Bentonite	35.0	Mg Bentonite	35.0
Glycerin	10.0	Distilled Water	65.0
Distilled Water	55.0		

Preparation of Bases.—The individual bentonites were added, in divided portions, to the glycerin and water, or water alone, in a mechanical mixer operated at a slow rate[1] of speed and stirred until a homogeneous preparation resulted.

Ointments of sulfathiazole (5% w/w), ammoniated mercury (5% w/w), boric acid (10% w/w), phenol (2% w/w), and iodine (7% w/w) were incorporated into each of the prepared bentonite bases, using proper pharmaceutical technique. Pharmaceutical necessities such as potassium iodide and iodine were used in the same concentrations as are found in iodine ointment, N. F. VIII, and phenol ointment, U. S. P. XIII.

Two methylcellulose bases, one containing glycerin, were also prepared according to the following formulas:

Methylcellulose Base

	w/w, %
Methylcellulose[2] (4,000 cps.)	5.00
Distilled Water	95.00

Methylcellulose Base with 10% Glycerin

	w/w, %
Methylcellulose[2] (4,000 cps.)	5.00
Glycerin	10.00
Distilled Water	85.00

Fifty cubic centimeters of hot water (80–90°) was added to the Methylcellulose in a beaker and mixed for five minutes, then allowed to stand for thirty minutes. Forty-five cubic centimeters of cold water (10 Gm. of glycerin and 35 cc. of cold water added in making the base-containing glycerin) was then added with constant stirring. A preparation of ointment-like consistency resulted.

Bacteriological tests were performed on the various ointments and bases using a modified F. D. A. cup-plate method. The modification, first utilized by Darlington (2), makes use of bromocresol purple indicator, which is added at the proportion of 25 ml. per 1,000 ml. of plating media, just before the pouring of the plates. The test organisms utilized in the tests were *Staphylococcus aureus*, *E. coli*, and *S. typhosa*.

* Received June 22, 1950, from the Pharmaceutical Research Laboratory, College of Pharmacy, The Ohio State University, Columbus.

† Abstract of part of a dissertation submitted by Martin Barr to the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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[1] Number one speed, Hamilton Beach Mixer, Model E.

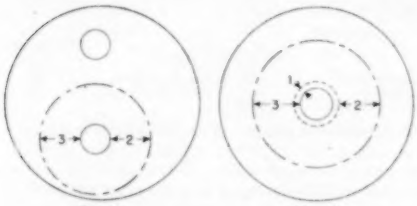
[2] Methocel, Dow Chemical Corporation.

RESULTS

The zones of inhibition produced by the various ointments and bases were similar to those reported in a previous paper (1), and are therefore not listed.

The production of acid zones of *p*H change was observed in the tests. These zones were colored yellow, indicating a *p*H of 5.2 or less with bromocresol purple indicator. These zones of *p*H change were produced only with the glycerin-containing ointments and bases.

Zones of apparent stimulation of growth of the bacteria were also observed upon testing the glycerin-containing ointments and bases. The results are listed in Table I. Figure 1 illustrates the location of these zones on the test plates.



Top cup—White Ointment (U. S. P. XIII); *bottom cup*—Volclay Bentonite Base. H Bentonite Base.

Fig. 1.—Cup plate tests on bentonite bases and white ointment.

Key: *1*—Zone of inhibition of growth; *2*—Zone of *p*H change; *3*—Zone of apparent stimulation of growth.

DISCUSSION

Darlington (2) showed that the use of bromocresol purple indicator in the bacteriological testing of ointments did not interfere with or alter the results obtained as when the same tests were carried out without indicator. Therefore, it was advantageous to utilize the indicator in the study of the bacteriological phenomena investigated in this study.

It is evident from the experimental results that the zone of *p*H change, observed upon testing the bentonite bases and ointments, occurs only when glycerin is present in such bases. It is suggested that the glycerin, diffusing from the tested ointments and bases into the agar media which is inoculated with the various test organisms, is acted on by the growing organisms during their metabolic processes. Acid oxidation products (possibly lactic acid or pyruvic acid), which lower the *p*H of the media in the acted area to *p*H 5.2 or below, may form. This acid *p*H accounts for the yellow color with bromocresol purple indicator. In order to emphasize that bentonites do not have a role in this phenomenon, it may be noted that the yellow acid zone of *p*H change was obtained upon testing the methyl cellulose base with glycerin, while this zone was not produced upon testing methyl cellulose base. This again indicates that it was the glycerin being acted on by the organisms producing acid products.

The sizes of the acid zones were found to vary slightly with each drug incorporated into the bases and with each of the test organisms. The zone size probably depends on the extent of diffusion of the glycerin from the ointment bases and on the ability of the organism to act on the glycerin. The largest zones were produced with *S. typhosa* and the smallest with *E. coli*. When drugs were incorporated into the bases, the size of the zones decreased. This may represent a decrease in the diffusion of the glycerin from the base into the media.

The zones of apparent stimulation of growth were obtained by testing the glycerin-containing ointments and bases against *E. coli* and *S. typhosa*, the former being stimulated in growth more than the latter. The zones of apparent stimulation of

TABLE I.—PRODUCTION OF ZONES OF *p*H CHANGE AND ZONES OF APPARENT STIMULATION OF GROWTH UPON TESTING GLYCERIN-CONTAINING BENTONITE BASES AND OINTMENTS WITH MEDIUM CONTAINING BROMOCRESOL PURPLE INDICATOR

5% Sulfathiazole in Base	*S. aureus* Zone of *p*H Change in Mm.[a]	*E. coli* Zone of *p*H Change in Mm.[a]	*S. typhosa* Zone of *p*H Change in Mm.[a]
White Ointment	0.0	0.0	0.0
1	20.0	15.0[c]	15.0[d]
2	20.0	15.0[c]	15.0[d]
3	20.0	15.0[c]	15.0[d]
4	20.0	15.0[c]	15.0[d]
5	20.0	15.0[c]	15.0[d]
6	20.0	15.0[c]	15.0[d]
5% Ammoniated Mercury in Base			
U. S. P. XIII	0.0	0.0	0.0
1	20.0	18.0[c]	20.0[d]
2	20.0	18.0[c]	20.0[d]
3	20.0	18.0[c]	20.0[d]
4	20.0	18.0[c]	20.0[d]
5	20.0	18.0[c]	20.0[d]
6	20.0	18.0[c]	20.0[d]
10% Boric Acid in Base			
U. S. P. XIII	0.0	0.0	0.0
1	16.0	16.0[c]	13.0[d]
2	16.0	16.0[c]	13.0[d]
3	16.0	16.0[c]	13.0[d]
4	16.0	16.0[c]	13.0[d]
5	16.0	16.0[c]	13.0[d]
6	16.0	16.0[c]	13.0[d]
2% Phenol in Base			
U. S. P. XIII	0.0	0.0	0.0[d]
1	20.0	17.0[c]	21.0[d]
2	20.0	17.0[c]	21.0[d]
3	20.0	17.0[c]	21.0[d]
4	20.0	17.0[c]	21.0[d]
5	20.0	17.0[c]	21.0[d]
6	20.0	17.0[c]	21.0[d]
4% Iodine; 4% KI in Base[b]			
Bentonite Bases			
White Ointment	0.0	0.0	0.0
1	22.0	18.0[c]	23.0[d]
2	22.0	18.0[c]	23.0[d]
3	22.0	18.0[c]	23.0[d]
4	22.0	18.0[c]	23.0[d]
5	22.0	18.0[c]	23.0[d]
6	22.0	18.0[c]	23.0[d]

[a] Zones of *p*H change were yellow in color.
[b] No acid zones or zones of apparent stimulation of growth noted.
[c] Zones of apparent stimulation of growth.
[d] Zones of slight stimulation of growth.

growth appeared only over the area where there was a zone of *p*H change. It is possible that some end products, formed as a result of the action of the growing organisms on the glycerin, may act as a growth stimulant to *E. coli* and *S. typhosa*. Since the zones of apparent stimulation of growth occur only over the area where there is a zone of *p*H change, it is logical to assume that some breakdown substance of glycerin is responsible for this phenomenon. The fact that *E. coli* is the most active fermenter of carbohydrates of the organisms utilized probably accounts for the greater stimulation of growth evidenced with this organism.

SUMMARY

1. In the study of the zone of *p*H change, it was apparent from the results that:

(*a*) The zone is a result of the action of the test organism on the glycerin diffusing from the ointments and bases, the formed acid products producing an acid zone of *p*H 5.2 or less.

(*b*) The size of the zone is influenced by the drug incorporated into the base.

(*c*) The size of the zone is influenced by the organism.

2. In the study of the zone of apparent stimulation of growth, it was evident that:

(*a*) There is more concentrated stimulatory zone produced with *E. coli* than with *S. typhosa*.

(*b*) The zone appears only where there is a zone of *p*H change.

(*c*) It is possible that the zones represent a stimulation of the metabolism of the growing organism by the breakdown products of the glycerin.

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Further Studies on the Alkaloids of *Argemone hispida**,†

By JOHN W. SCHERMERHORN‡ and TAITO O. SOINE§

Further structural studies have been carried out on two new alkaloids isolated from *Argemone hispida*. The name "argemonine" has been recommended for the alkaloid melting, when dried, at 152.5 to 153°. "Norargemonine" has been recommended as the name for the alkaloid melting at 238°. The data reported serve to add to the knowledge of the structure of argemonine and norargemonine but do not enable the writing of structural formulas at the present time.

IN A PREVIOUS phytochemical study of *Argemone hispida*, Soine and Gisvold (1) have shown that it contains, among other things, a series of alkaloids. A review of the literature has shown that they have not been separated previously and may, therefore, be considered as new compounds.

In view of the fact that the two alkaloids isolated in pure form are new compounds, and in order to simplify subsequent nomenclature, it has been deemed advisable to assign them names at this time. The name "argemonine" was assigned by Schlotterbeck (2) to an alkaloid isolated from *Argemone Mexicana*. Subsequent investigation has shown this alkaloid to be protopine. It is now recommended that the name "argemonine" be applied to the nonphenolic alkaloid isolated from *A. hispida* which melts, after drying, at 153°.[1]

"Norargemonine" is recommended as the name for the mono-phenolic alkaloid isolated from this plant and melting at 238°. This follows accepted rules of nomenclature in that the empirical formula of this compound contains the equivalent of one less methylene group than argemonine.

Examination of a fresh batch of plant, collected in the same area but at a slightly earlier stage of development than the original sample, resulted in the isolation of both argemonine and norargemonine. No trace was found of the amorphous

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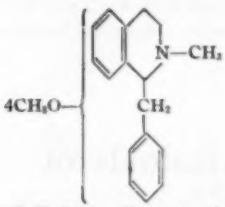
[1] All melting points in this work are uncorrected.

gelatinous, alkaloidal fraction reported in the previous work(1).

Examination of Argemonine.—In view of the fact that the plant is a member of the *Papaveraceæ*, and because only isoquinoline-type alkaloids have been isolated from this family, it was felt that argemonine probably also contained some type of isoquinoline nucleus. N-methyl determinations showed the presence of an N-methyl group, so the empirical formula could be written as follows: $C_{18}H_{10}(OCH_3)_4N(CH_3)$. Should the alkaloid prove to have a benzylisoquinoline structure of the papaverine type, it would have to contain a double bond in addition to the two aromatic nuclei in order to satisfy the analytical data. In order to account for the optical activity of the



$C_{21}H_{27}O_4N$ or H_2 too much

compound, the double bond could only be at position 3,4 and should be easily reduced or brominated. Neither of these treatments was successful. Another fact tending to disprove the benzylisoquinoline formula was the failure to obtain isoquinoline on zinc dust distillation. A basic dehydrogenation product was obtained but could not be identified.

Potassium permanganate oxidation yielded, as the only identifiable product, metahemipinic acid, suggesting that the four methoxyls are equally distributed between the two aromatic nuclei and that in at least one of the phenyl rings they are adjacent.

Manganese dioxide oxidation yielded, as the only crystalline fragment, an unidentified, basic compound possessing no carboxyl groups. Attempts to demonstrate the presence of an aldehyde or ketone group were unsuccessful. Insufficient material was available to carry out analytical determinations.

An infrared examination of a solution of the alkaloid in carbon disulfide was carried out.

*p*K determinations, performed in an effort to determine the relative basicity of the nitrogen, were somewhat inconclusive.

Norargemonine.—In order to compare this alkaloid more closely with argemonine, it was treated with diazomethane to methylate the free phenolic group. Examination of the resulting product proved it to be identical with argemonine, showing norargemonine to differ from it only in the absence of one O-methyl group.

Several attempts to form a methiodide failed, even though the nitrogen was known to be tertiary. In addition, infrared examination failed to reveal the characteristic absorption peak for hydroxyls, although one such group was known to be present.

A possible explanation for these phenomena is hydrogen bonding, which is known to prevent the hydroxyl absorption peak from appearing during infrared examination and could conceivably interfere with methiodide formation. Hendricks, *et al.* (4) have pointed out that such bonding is not uncommon and that it causes almost complete obliteration of the hydroxyl absorption peak.

While no structural formula can be written from the information now at hand, it would appear that both alkaloids could possibly possess a fused, four-ring system containing a tetrahydroisoquinoline and benzene nucleus. A system of this type would satisfy all the analytical data and is not uncommon in the *Papaveraceæ*.

EXPERIMENTAL

The crude drug in this work was obtained from the same general vicinity as that used in the previous work, but a gross examination revealed that it had been collected at a somewhat earlier stage of development. The seed pods of the original lot were bursting, while those in the present sample were in the early stages of formation.

The drug was reduced to a No. 20 powder and exhausted of alkaloid by percolation with a menstruum of 2% tartaric acid in 95% alcohol. The entire percolate was then evaporated to a thick tar under reduced pressure at temperatures not exceeding 55°.

The tarry residue was freed of alkaloid by repeated extractions with water and the alkaloid tartrates precipitated from aqueous solution by the addition of sodium carbonate. The liberated alkaloids were extracted with chloroform and the chloroformic extract was evaporated to dryness in order to remove any residual alcohol from the original percolation.

The residue was extracted with 5% acetic acid until all the alkaloidal material had been removed. In order to remove more impurities, this aqueous acid extract was washed with chloroform. The washings were found to be alkaloidal in nature and subsequent investigation revealed the material to be identical with norargemonine.

The aqueous solution of alkaloid acetates was made alkaline with 10% sodium hydroxide and extracted with ether until the ether extracts were free of alkaloid.

The sodium hydroxide-insoluble alkaloids were partitioned several times between ether and 5% aqueous acetic acid, the final ether extract being dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by several recrystallizations from a methanol-water solution, and yielded a crystalline alkaloid identical with argemonine.

The solution of sodium hydroxide-soluble alkaloids was made acid with acetic acid and then alkalinized with sodium carbonate. This alkaline solution was extracted with chloroform until all alkaloidal material had been removed. The alkaloids were partitioned several times between chloroform and 5% aqueous acetic acid, the final chloroformic extract being dried over anhydrous sodium sulfate and evaporated to dryness. The residue was purified by several recrystallizations from methanol, yielding an alkaloid identical with norargemonine.

Careful examination of all fractions failed to demonstrate the presence of the previously reported (1) amorphous, gelatinous alkaloidal fraction.

Examination of the Argemonine

N-Methyl Content.—Volumetric N-methyl determinations depending on the liberation of methyl iodide demonstrated the presence of an N-methyl group.

Anal.—Calcd. for $C_{21}H_{23}O_4N$: 4.23%. Found: (average) 4.68%.

Zinc Dust Distillation (5).—One gram of the alkaloid was heated at 450° for ten minutes with 10 Gm. of reagent quality zinc dust. The vapors were carried off in a stream of nitrogen and condensed in an ice-cooled container. The residue in the tube was extracted with ether and the ether extract exhausted of acid-soluble material with 5% hydrochloric acid. The collection tube was washed with 5% hydrochloric acid and the two extracts were combined, alkalinized with sodium carbonate, and extracted with ether. The ether extract was dried over anhydrous sodium sulfate and treated with a saturated solution of picric acid in anhydrous ether. An immediate precipitate formed which could be only partially purified by recrystallization. This material softened to a reddish glass at about 120° and melted at 217–220°. A mixed melting-point determination with an authentic sample of isoquinoline picrate softened at 200–205° and melted at 208–213°.

Bromination.—One hundred milligrams of alkaloid was dissolved in 5 cc. of carbon tetrachloride, and a solution of bromine in carbon tetrachloride added until there was no further decolorization. An immediate precipitate formed which would not redissolve.

The precipitated material was completely soluble in water; a few drops of the aqueous solution giving a very slight precipitate with silver nitrate test solution. Isolation of the product gave rise only to a compound identical with argemonine.

Reduction Studies.—Four hundred and fifty milligrams of alkaloid was dissolved in 50 cc. of alcohol and the solution was shaken for three hours with 50 mg. of platinum oxide under an atmosphere of 40 lb. of hydrogen. The original alkaloid was isolated unchanged.

pK Determinations.—A 0.0015 *M* solution of argemonine hydrochloride in a mixture of 50 cc. of methanol and 100 cc. of distilled water was used. The controls were all of similar molarity and in the same solvent. A Cenco electric titration meter and Beckman glass electrodes were used.

Compound	pK Value	Reported (3)
1,2,3,4-Tetrahydroisoquinoline HCl	4.55	5.03
Isoquinoline HCl	4.99	5.38
Papaverine HCl	5.50	
Argemonine HCl	6.65	

Potassium Permanganate Oxidation.—One gram of the alkaloid was dissolved in a mixture of 2.5 cc. of hydrochloric acid and 35 cc. of hot water. The alkaloidal salt was decomposed by the addition of 6.8 Gm. of potassium carbonate. The mixture was heated to boiling and a 5% aqueous solution of potassium permanganate added slowly until the color was no longer discharged. Approximately 3 Gm. of permanganate was required.

The oxidation mixture was filtered, acidified, and subjected to continuous ether extraction in a liquid-liquid extractor for several days. The ether was removed from the extract and the residue subjected to fractional distillation. All of the liquid came over between 75° and 100°, and was shown to be alcohol and water, contaminants of the ether. When the last of the liquid had passed over, some sublimation of the residue was noted. This material was dissolved in boiling water, filtered, and set aside. No crystallization occurred, so it was partitioned back into ether and the ether was allowed to evaporate spontaneously. A number of long, brown-colored needles separated and were found to soften at 140° and to melt at 155–160° with effervescence. Efforts to purify by recrystallization failed. A 30-mg. portion of the material was subjected to microsublimation, giving an almost white compound melting at 172–173°, corresponding to metahemipinic acid anhydride.

A second 30-mg. portion of the crude material was dissolved in an excess of a solution of 33% ethyl amine in water. The solution was evaporated to dryness and sublimed at 15 mm. pressure. The sublimed material melted at 225–227°, corresponding to the ethyl imide of metahemipinic acid.

The acid-soluble portion of the permanganate oxidation yielded a small amount of a tarry substance which could not be purified.

Manganese Dioxide Oxidation.—One gram of the alkaloid was dissolved in a mixture of 2.5 cc. of concentrated sulfuric acid and 100 cc. of distilled water. One gram of manganese dioxide was added to the solution and the mixture was stirred mechanically on a boiling water bath for three hours.

Continuous extraction of the acidic oxidation mixture with ether for five days yielded only a tar which could not be purified.

The acidic solution, after removal of acid-insoluble material, was alkalinized with sodium carbonate and extracted continuously with ether for four days. The ether extract was dried and the ether evaporated, leaving a small quantity of a brown, tarry substance. This material was recrystallized from alcohol-water; the resulting compound melting at 116–118°.

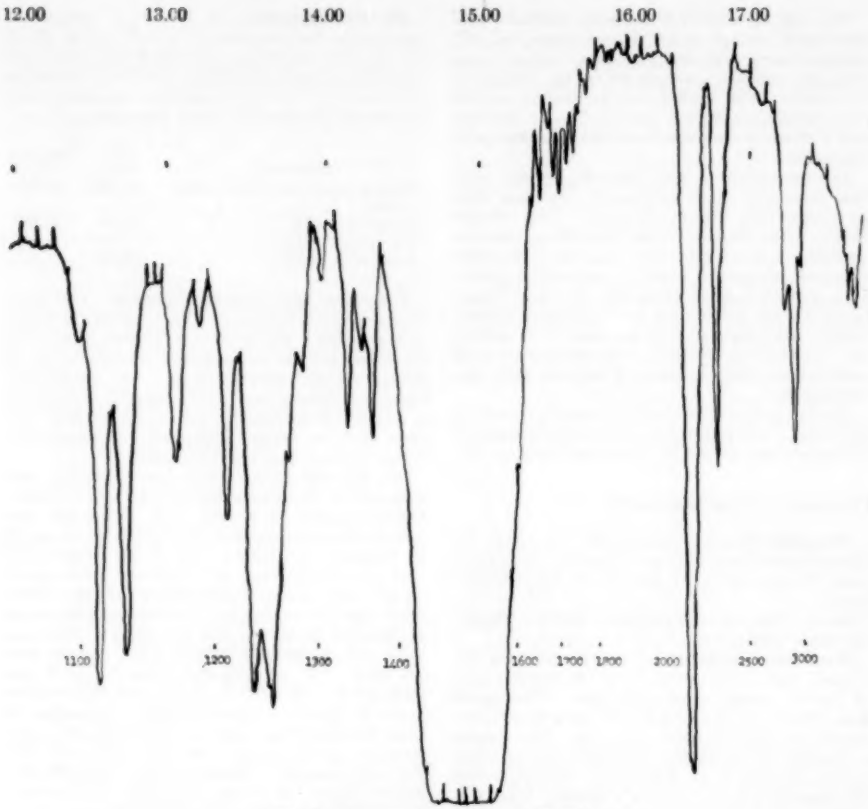


Fig. 1.—Argemonine in carbon disulfide.

Treatment of Oxidation Product.—*Solubility.*—The material was insoluble in water, 5% sodium hydroxide, and 5% sodium bicarbonate. It dissolved in 5% hydrochloric acid and the usual organic solvents.

Sodium Fusion.—Sodium fusion gave a positive test for nitrogen.

Derivatives.—No sodium bisulfite addition product or 2,4-dinitrophenylhydrazone could be prepared. A picrate was prepared which melted at 212–215°. Insufficient material remained for any other analyses.

The oxidation mixture, after removal of the acid and basic portions, was titrated to exact neutrality and extracted for four days with ether to remove any amphoteric material. Only a very small portion of a tan, gummy material could be obtained, which could not be worked up.

Infrared Examination.[3,4]—An infrared examination of a solution of the alkaloid in carbon disulfide was made. A portion of the spectrogram is reproduced in Fig. 1.

Treatment of Norargemonine

Specific Rotation.—The specific rotation of a sample of 0.2786 Gm. of the alkaloid in 10 cc. of chloroform was $[\alpha]_D^{27} = -153.69°$, as compared with the previously reported value of $[\alpha]_D^{25} = -77.47°$ (1).

Infrared Examination.—An infrared examination of a sample of the crystalline alkaloid was made. The characteristic absorption peak for free hydroxyl groups was absent. A portion of the spectrogram is reproduced in Fig. 2.

Derivatives.—Several attempts were made to prepare the methiodide, with no success. In every case the unchanged alkaloid was recovered from the reaction mixture.

One hundred milligrams of norargemonine was dissolved in 20 cc. of absolute methanol and an ether solution of diazomethane added slowly until an excess remained. The resultant product was insoluble in 10% sodium hydroxide, indicating the absence of the free phenolic group. Recrystal-

[3] The authors wish to acknowledge the kind assistance of Dr. Bryce L. Crawford of the Department of Chemistry, University of Minnesota, who carried out the infrared examinations in this work and interpreted the results.

[4] A Perkin-Elmer infrared spectrometer, Model 12-C, was used in this work. The samples were examined in a 100 μ cell.

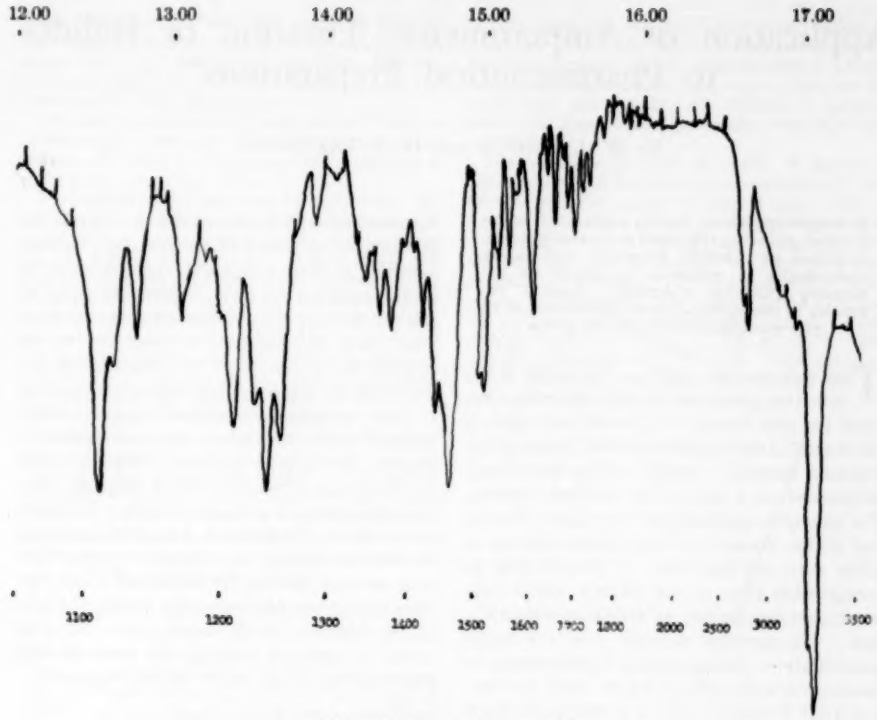


Fig. 2.—Norargemonine crystals.

lization and drying gave a compound melting at 149.5 to 151.5°. When mixed with an authentic sample of argemonine, there was no depression of melting point.

SUMMARY

1. Further structural studies have been carried out on a nonphenolic and a mono-phenolic alkaloid isolated from *Argemone hispida*. It has been recommended that they be called argemonine and norargemonine, respectively.

2. Structural studies on argemonine have revealed the following data:

(*a*) The alkaloid contains an N-methyl group.

(*b*) The alkaloid contains no active unsaturated centers in the molecule.

(*c*) Two of the methoxyl groups are adjacent and are in one of the two benzene nuclei present in the molecule.

(*d*) There is no unsaturation in the nitrogen ring of the compound.

3. Structural studies on norargemonine have revealed the following data:

(*a*) It differs from argemonine in that it contains three methoxyl and one phenolic group, instead of four methoxyl groups.

(*b*) There appears to be hydrogen bonding between the phenolic hydroxyl group and the tertiary nitrogen.

(*c*) The previously reported specific rotation of $[\alpha]_D^{24} = -77.47°$ (chloroform) has been corrected to $[\alpha]_D^{27} = -153.69°$ (chloroform).

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Application of Amperometric Titration of Halides to Pharmaceutical Preparations*

By W. J. MADER and H. A. FREDIANI

A simple apparatus, readily assembled, is described, allowing the rapid and precise determination of chloride, bromide, and iodide individually in mixtures by means of the rotating platinum electrode. Several examples of the application of this technique to pharmaceutical products are given.

THE amperometric method for halide determination using the rotating platinum electrode has been found to be precise and rapid for the control of many pharmaceutical preparations. Chloride, bromide, or iodide may be individually determined on a mixture of the three halides. The method is applicable to very dilute solutions and in the presence of large concentrations of acids, salts, and insolubles. A titration may be completed in three to five minutes, and a technician may run as many as 50 determinations in a day. The accuracy depends upon the halide concentration; for example 0.1 *N* chloride may be determined with an accuracy of ±0.1 per cent and 0.001 *N* chloride with an accuracy of ±3 per cent.

In amperometric titrations as defined by Kolthoff (1, 2), the current which passes through the titration cell between an indicator electrode and an appropriate depolarized reference electrode at a suitable applied E. M. F. is measured as a function of the volume of a suitable titrating solution. Unlike polarography, wherein the increase in current is measured and recorded under conditions of constant concentration of reducible substance and continuously changing applied potential, in amperometric titrations the applied potential is maintained at a constant value, greater than the decomposition potential of the reducible ion, and the change in current recorded upon increasing concentration of reducible ion. The application of amperometric titrations to the determination of chloride, bromide, and iodide has recently been reported (3, 4). The current measured is directly related to the concentration of excess silver ion present. The current passing through the indicator electrode or the rotating platinum electrode and a calomel or Kolthoff depolarized reference electrode is measured most conveniently with a microammeter. In this application the potential of the rotating platinum electrode is made sufficiently negative so as to cause deposition of silver, leaving the layer of solution next to the electrode depleted of silver ions. It is the migration of these ions to the cathode that gives rise to the diffusion current. The applied potential is not sufficiently negative to cause reduction of dissolved oxygen. Silver bromide and silver iodide do not cathodically depolarize the rotating platinum electrode under the conditions employed. Silver chloride, however, does cause a cathodic current. To determine iodide in the presence of bromide, ammonia is added to increase the solubility of silver bromide without affecting the iodide end point. All three halides can be successively titrated by first adding ammonia for the iodide, then adding an excess of acid and titrating the bromide, and finally adding gelatin for the chloride endpoint.

APPARATUS

The apparatus (Fig. 1) consists of an "H" type titration cell connected by means of an agar-potassium nitrate bridge to the reference electrode. The rotating platinum electrode (400–700 r. p. m.), which is powered by a Sargent cone drive motor, is connected to the positive pole of a microammeter and the reference electrode is connected to the negative post.

The platinum electrode is prepared by sealing a sturdy piece of No. 18 platinum wire into 6-mm. soft glass tubing to form an electrode 5–10 mm.

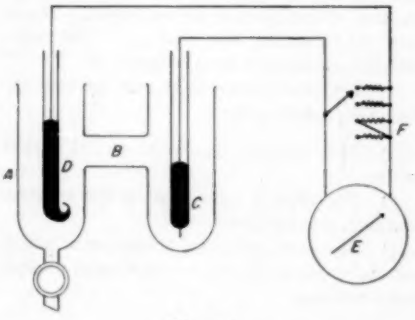

Figure 1.

A, titration cell; *B*, agar potassium nitrate bridge; *C*, reference electrode; *D*, rotating platinum electrode; *E*, microammeter; *F*, ayrton shunt.

* Received May 5, 1950, from Merck and Co., Inc., Rahway, N. J.
Presented to the Scientific Section, A. PH. A., Atlantic City meeting, May, 1950.

long. The platinum wire and tubing is bent at right angles and about circular 2 cm. from the end, away from the direction of rotation. Mercury inside the tube serves to make contact between the platinum and the copper wire.

The microammeter has a range of 0–100 microamperes with 50 scale divisions. A system of resistors as shown in Fig. 1 permits its use over a wider range of current values.

Two reference electrodes are in general use, the saturated calomel and the Kolthoff cell; the former being used for chloride and bromide, and the latter for the iodide determination. The calomel cell is prepared in the usual manner by placing a layer of mercury in the bottom of the cell followed by a layer of calomel paste, in turn followed by a saturated solution of potassium chloride. The Kolthoff cell is prepared by adding mercury to the cell followed by a solution of 4.2 Gm. of potassium iodide and 1.3 Gm. mercuric iodide in 100 ml. of saturated potassium chloride solution.

The agar bridge is prepared by adding 3 Gm. of agar to 100 ml. of boiling distilled water. To this is added 18 Gm. of potassium nitrate. The mixture is brought to boil and heating continued for several minutes. A cork is placed in the reference electrode side of the bridge and the hot agar added from the other end. After cooling, the cork is removed and agar added to complete the bridge.

PROCEDURE

General

General procedures are given for the determination of chloride using 0.1 *N*, 0.01 *N*, and 0.001 *N* silver nitrate and for the simultaneous determination of iodide, bromide, and chloride.

Determination of Chloride, 0.1 *N* and 0.01 *N* $AgNO_3$.—Chloride in the concentration range 35–100 mg. is titrated with 0.1 *N* $AgNO_3$. For the 1–35 mg. range, 0.01 *N* $AgNO_3$ is used. The sample is dissolved in 100 ml. of water, acidified with 3 ml. of concentrated nitric acid, and 10 ml. of 1% gelatin added. The solution is titrated with 0.1 *N* $AgNO_3$, measuring the residual current before adding silver nitrate. The silver nitrate is added rapidly until the needle of the meter begins to fluctuate. The silver nitrate is then added slowly, recording several of the current-volume points, namely the original point and two or three on the steep slope (see Fig. 2). No attempt is made to obtain readings in the immediate vicinity of the end point.

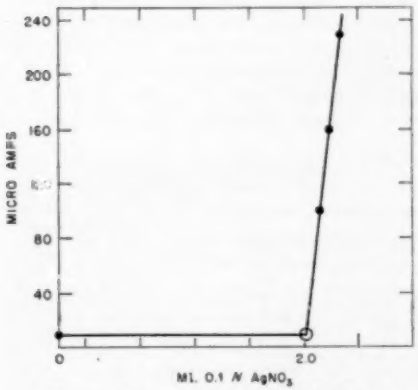

Fig. 2.—Titration of 2.0 ml. 0.1 *N* NaCl.

Determination of Chloride, 0.001 *N* $AgNO_3$.—When chloride is present in concentrations of less than 1 mg., the sample is titrated with 0.001 *N* $AgNO_3$. The same general procedure is used as in the titration with 0.1 *N* and 0.01 *N* $AgNO_3$, with the exception that the sample is dissolved or suspended in 100 ml. of 75% acetone, 0.8 *N* with respect to nitric acid. The acetone serves to reduce the solubility of the silver chloride formed. In all chloride titrations, 10 ml. of 1% gelatin is used. The data are plotted as in Fig. 3. High accuracy on smaller samples is possible by carrying out the titration in a smaller volume of solvent.

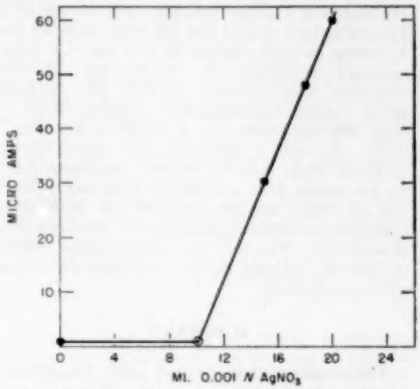

Fig. 3.—Titration of 10 ml. 0.001 *N* NaCl.

Simultaneous Determination of Iodide, Bromide, and Chloride.—The solution containing the three halides in the concentration range 0.01 to 0.0001 *N* for all three is added to the titration cell and diluted to 100 ml. with water. Add 1 ml. of concentrated ammonia. Use the Kolthoff reference electrode. When the needle remains constant, note the current and add 0.01 *N* silver nitrate rapidly until the needle begins to fluctuate. Then add the silver nitrate slowly, observing several current-volume points. When the current reaches 90–100 microamperes, neutralize the solution with concentrated nitric acid and add 1 ml. excess. Change from the Kolthoff reference electrode to the saturated calomel electrode and, after the current is constant, titrate the bromide. After passing the bromide end point, add 10 ml. 1% gelatin and titrate the chloride. A typical graph is shown in Fig. 4.

In this example, the following results were obtained:

0.01 *N*, Ml.	Halide Added	Found, Ml.	%
6.00	KI	6.02	100.2
5.00	NaBr	4.88	97.6
6.00	NaCl	5.85	97.5

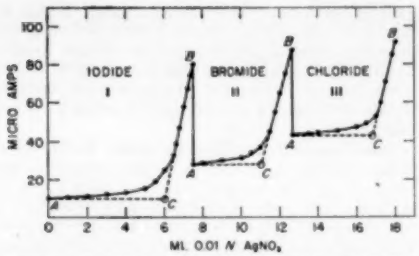

Fig. 4.—Titration of bromide, chloride and iodide.

The pharmaceutical products which are tested for halide by the amperometric method in the Merck Laboratories are too numerous to mention completely. However, a few, illustrative of the precision and accuracy of the method, will be given. In analyzing a saline solution, the average of six determinations carried out on 1-ml. samples gave a sodium chloride content of 9.01 mg./ml. with a standard deviation of ±0.06 mg. Mechlorethamine hydrochloride, [methyl bis (β-chloroethyl) amine hydrochloride] assayed 99.6% on eight determinations with a standard deviation of 0.1%. Flaxedil, a curarizing agent $C_6H_3[OC_2H_4\text{-}N(C_2H_5)_2IC_2H_5]_2$, may be titrated amperometrically for the iodide with a standard deviation of 0.1%, and its saline solution analyzed for both active ingredient and sodium chloride with the same order of accuracy. The salt content of a saline solution of Cobione (vitamin B_{12}) has also been determined amperometrically.

SUMMARY

The amperometric method using the rotating platinum electrode has been found to be an excellent procedure for the control of pharmaceutical preparations by means of halide determinations, where applicable. Chloride, bromide, or iodide can be individually determined on a mixture of the three halides. The accuracy of the method depends upon the halide concentration, e. g., 0.1 *N* chloride may be determined with an accuracy of ±0.1 per cent and 10^{-4} *N* chloride 3 per cent. The apparatus consists of an H-type cell containing a standard calomel electrode on the one side connected to the titration section by means of an agar-potassium nitrate bridge. A rotating platinum electrode is used and the cathode and anode connected to a microammeter with suitable shunts inserted. To determine chloride, the solution acidified with nitric acid is added to the cell, gelatin is used as maximum suppressor and the solution titrated with standard silver nitrate. The end point is determined by plotting volume silver nitrate against microamperes. This method is rapid; a technician can run as high as 50 determinations in a day. Examples are included of the determination of chloride, bromide, and iodide in pharmaceutical preparations in which the halides appear singly and in combination. Compared with the Volhard method, the amperometric method has the obvious advantage of a direct titration over a back titration method. The amperometric method also has the advantage over the Mohr and Fajans methods in its applicability at high dilutions and in acid or alkaline medium.

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A Collaborative Study on the Acute Toxicity Testing of Several Drugs*,†

By M. G. ALLMARK‡,§

ALMOST everyone has observed the great difference between laboratories on the reported toxicities of drugs. These differences are due to many causes, such as variations in technique employed, seasonal and individual variations in animals, weight range used, etc. It has become a bit disconcerting at times to notice the discrepancies in reports on the toxicity of a new drug.

Knowing these facts, the Committee on Physiological Testing decided in favor of a collaborative study on the acute toxicity testing of drugs. The principal objective of this study was to determine the acute toxicity of a few well-known drugs under conditions as nearly identical as possible and by accepted methods to analyze the data in order to find out: (*a*) if significant variations in the LD_{50}'s for the same drugs existed between laboratories; (*b*) if the estimates of relative toxicities from individual assays for similar-acting drugs were the same or different for all the laboratories; (*c*) if the data would show that it would be possible and preferable to report the toxicity of a new drug in terms of a drug of similar action and properties.

The following drugs were sent to each collaborator: Benadryl, Pyribenzamine, sodium phenobarbital, and sodium pentobarbital.

Eight laboratories took part in this study: Bristol Laboratories, Syracuse, N. Y.; Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, Lincoln, Nebr.; Eli Lilly and Co., Indianapolis, Ind.; Sterling-Winthrop Research Institute, Rensselaer, N. Y.; Parke, Davis and Co., Detroit, Mich.; Munch Research Laboratories, Upper Darby, Pa.; Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada; and the Upjohn Co., Kalamazoo, Mich.

* Received October 27, 1948, from the Department of National Health and Welfare, Ottawa, Canada.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.
† Report of the A. PH. A. Committee on Physiological Testing, 1947–1948.
‡ Chairman of the A. PH. A. Committee on Physiological Testing, 1947–1948. Present address: Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Canada.
§ The Department of Physiology and Pharmacology, College of Pharmacy, University of Nebraska, wishes to acknowledge the support by grants from the University of Nebraska Research Council. As chairman, Mr. Allmark wishes to thank the various members of the committee for their splendid cooperation; Elizabeth Carmichael and George Taker for assistance in computing and compiling the data; Parke, Davis & Co. and Ciba Company for a generous supply of Benadryl and Pyribenzamine.

PROCEDURE

General Directions for Carrying Out the Acute Toxicity Tests

Selection of Animals.—Use healthy, unstarved, male mice 17–22 Gm. in weight. Weigh to the nearest 0.5 Gm. Arrange the mice so that the average weight in each group will be the same.

Preparation of Solutions.—Dissolve the required amount of drug in distilled water just before the injections are made. Due to the differences in sensitivity of mice colonies, the concentration of each drug solution used in each laboratory could not be the same. For any one drug, a solution of the same concentration for all the doses selected could be used, or a different solution concentration for each dose could be used in order to maintain approximately the same volume injected into each mouse. For these tests the latter procedure will be adopted. Vary the concentration of solution for each dose so that the volume injected will be approximately 0.5 ± 0.1 cc.

Injection of Animals.—Use a tuberculin syringe of 1-cc. capacity, graduated in 0.01 cc., and fitted with a $^{5}/_{8}$ in. 25-gauge needle. Inject each mouse intraperitoneally with a dose estimated on the basis of milligrams of drug per kilogram of body weight. Place mice in individual cages after injection. The injections for each pair of drugs are made in a randomized order, e. g., the doses for Benadryl and Pyribenzamine should be made up at the same time and injected in a random order. The total number of deaths for each dose of Benadryl and Pyribenzamine is recorded for twenty-four hours following injections; for sodium phenobarbital and sodium pentobarbital, forty-eight hours.

Selection of Doses.—Select doses so that mortalities of approximately 20–80% will result. Inject four groups of 15 mice for each drug. If it is not possible to inject 15 mice on each dose, reduce the number to 10. The interval between doses should be the same, i. e., the doses selected should be such that their logarithms are equally spaced.

Other Directions.—Randomize the order of injecting the respective doses by a device such as shaking cardboard tags, corresponding to the doses, in a box. The temperature of the animal room should be between 67–77° F., if possible. The solutions should be at room temperature when injected. Report deaths for barbiturates after twenty-four, forty-eight, and seventy-two hours.

RESULTS AND DISCUSSION

Tables I and II show a compilation of the data. The methods of Bliss (1, 2) and Laug, *et al.* (3), have been used in computing the various values given in the tables. The calculations for the bar-

TABLE I.—COMPILED DATA OF SODIUM PHENOBARBITAL AND SODIUM PENTOBARBITAL TOXICITY DETERMINATIONS

1 Lab. No.	2 Date of Assay	3 Product	4 Dose, Mg./Kg.	5 Mort., %	6 χ^2	7 b, Slope of D. M. Curves	8 χ^2_{bb}	9 LD$_{50}$, Mg./Kg.	10 Actual Limits 1.96 S. E.	11 Composite Slope, ±S. E.	12 Observed Toxicity, %	13 Actual Limits 1.96 S. E.	14 Strain of Mice	15 Time in Lab. Before Test	16 Remarks
1	3/23/48	Sod. Ph.	220.0	0.0	1.77	23.61	0.10	253	243–262	22.66 ± 3.0	201	190–212	Unknown	2 days	
			246.4	46.7											
			276.0	86.7											
			309.0	93.3											
		Sod. P.	115.0	13.3	2.25	21.74	...	126	120–131						
			128.8	73.3											
			144.3	86.7											
			161.6	100.0											
2	4/30/48	Sod. Ph.	215.0	53.3	1.96	24.04	0.62	215	204–227	27.72 ± 6.5	178	169–187	Rockland Farms	1–3 wks.	
			227.0	60.0											
			239.7	93.3											
			253.0	93.3											
		Sod. P.	115.0	20.0	3.26	34.82	...	122	119–124						
			119.0	26.7											
			123.1	73.3											
			127.4	66.7											
3	5/8/48	Sod. Ph.	200.0	6.7	3.32	15.03	2.27	269	257–281	17.23 ± 1.9	200	189–213	White Swiss	1–3 wks.	
			220.0	13.3											
			242.0	13.3											
			266.2	46.7											
			292.6	66.7											
			321.9	93.3											
		Sod. P.	100.0	6.7	6.08	21.01	...	133	128–138						
			110.0	6.7											
			121.0	0.0											
			133.1	46.7											
			146.4	86.7											
			161.1	100.0											
4	4/6/48	Sod. Ph.	200.0	10 0	4.08	11.84	1.94	279	260–299	13.82 ± 2.3	201	183–221	Harlan	1 day	
			225.0	20.0											
			250.0	20.0											
			275.0	30.0											
			300.0	60.0											
			330.0	100.0											
		Sod. P.	110.0	10.0	3.16	19.05	...	136	129–144						
			125.0	10.0											
			140.0	70.0											
			160.0	90.0											
6	3/10/48	Sod. Ph.	175.0	20.0	2.12	13.63	2.84	205	193–218	16.43 ± 2.5	153	142–164	Tilden-James	1 day	Not injected at same time
			200.0	46.7											
			228.6	60.0											
			261.2	100.0											
	3/15/48	Sod. P.	110.0	6.7	2.27	22.90	...	134	129–139						
			121.0	13.3											
			133.0	33.3											
			146.4	86.7											
8	4/6/48	Sod. Ph.	218.8	0.0	4.31	27.48	0.13	265	256–274	25.91 ± 3.3	194	185–204	White Swiss	9 days	
			239.9	6.7											
			263.0	66.7											
			288.4	73.3											
		Sod. P.	109.6	0.0	0.17	25.04	...	137	132–141						
			120.2	6.7											
			131.8	40.0											
			144.5	73.3											
			158.5	93.3											

NOTE: Sod. Ph. means sodium phenobarbital; Sod. P. means sodium pentobarbital.

TABLE II.—COMPILED DATA OF BENADRYL AND PYRIBENZAMINE TOXICITY DETERMINATIONS

1 Lab. No.	2 Date of Assay	3 Product	4 Dose, Mg./Kg.	5 Mort., %	6 χ^2	7 b, Slope of D. M. Curves	8 χb^2	9 LD_{50} Mg./Kg.	10 Actual Limits, 1.96 S. E.	11 Composite Slope, ±S. E.	12 Observed Toxicity, %	13 Actual Limits, 1.96 S. E.	14 Strain of Mice	15 Time in Lab. Before Test	16 Remarks
1	3/4/48	B	82.67	3.3	0.19	11.67	0.34	117	108-128	12.96 ± 2.2	137	124-152	Unknown	2 days	
			94.91	13.3											
			108.97	40.0											
			125.12	60.0											
		P	74.63	13.3	2.36	14.27	...	85	80-91						
			85.69	66.7											
			98.38	80.0											
			112.95	93.3											
2	4/23/48	B	90.00	6.7	0.66	15.26	0.01	115	108-122	15.57 ± 2.5	144	133-156	Rockland Farms	3-4 wks.	
			100.00	20.0											
			111.20	33.3											
			123.50	73.3											
		P	60.00	0.0	0.22	15.76	...	80	75-84						
			70.00	20.0											
			81.66	60.0											
			95.26	86.7											
3	3/19/48	B	70.70	13.3	1.29	19.63	0.00	80	76-84	19.56 ± 2.9	141	132-150	White Swiss	1-2 wks.	
			79.40	46.7											
			89.10	73.3											
			100.00	93.3											
		P	50.00	20.0	0.83	19.49	...	57	54-59						
			56.10	40.0											
			63.00	80.0											
			70.70	100.0											
4	4/27/48	B	62.00	0.0	0.55	17.84	3.57	81	72-84	13.33 ± 1.72	120	111-130	Harlan	1 day	
			70.00	13.3											
			80.00	53.3											
			90.00	80.0											
			100.00	100.0											
		P	50.00	6.7	2.75	10.97	...	67	63-71						
			56.00	33.3											
			62.00	33.3											
			70.00	46.7											
			80.00	80.0											
			90.00	66.7											
5	4/13/48	B	50.00	0.0	0.76	7.70	15.51	80	71-89	9.80 ± 1.6	112	98-127	Dr. Webster's Strain of Swiss Mice	Not reported	Not injected at same time
			63.00	26.7											
			79.60	53.3											
			100.00	73.3											
	4/28/48	P	57.70	0.0	5.71	28.48	...	71	69-73						
			65.00	13.3											
			73.00	80.0											
			81.70	86.7											
6	3/25/48	B	60.00	26.7	1.99	8.51	0.28	74	67-81	9.32 ± 2.2	129	114-147	Tilden-James	1 day	Not injected at same time
			69.00	40.0											
			79.35	46.7											
			91.20	86.7											
	3/23/48	P	50.00	33.3	1.05	11.03	..	57	53-62						
			55.00	33.3											
			66.50	80.0											
7	4/30/48	B	60.00	0.0	0.57	11.82	0.46	105	96-116	10.57 ± 1.7	140	117-167	Not reported	1-2 wks.	
			80.00	10.0											
			105.00	40.0											
			135.00	100.0											
		P	45.00	0.0	0.18	9.45	...	76	65-89						
			60.00	20.0											
			80.00	100.0											
			105.00	90.0											
8	3/26/48	B	70.79	33.3	0.59	5.92	4.34	89	77-102	10.24 ± 2.3	158	141-178	White Swiss	23 days	Composite curve shows slight departure from parallelism
			79.43	33.3											
			89.13	46.7											
			100.00	66.7											
		P	50.12	26.7	0.75	15.52	...	56	53-59						
			56.23	46.7											
			63.10	73.3											
			70.79	100.0											

NOTE: B means Benadryl, and P means Pyribenzamine.

biturates were based on a forty-eight hour observation period. The deaths occurring after forty-eight hours were rather sporadic and, therefore, were not included.

Before commenting on the actual computations given in the tables, which were the main objectives of this study, it is of interest to note some of the other results.

From the data submitted by each collaborator, dosage-mortality curves were plotted as described by Bliss (1, 2). The *chi*-square (χ^2) values are given in column 6, Tables I and II. In every case the provisional regression lines were well fitted to the experimental points. The differences in slopes of these dosage-mortality curves are quite large; although not significantly different within laboratories for each pair of drugs (except a slight departure from parallelism from laboratory No. 8, Table II), they are significantly different between laboratories in a number of cases. For sodium phenobarbital the slopes varied from 11.84 to 24.04; sodium pentobarbital 15.03 to 34.82; Benadryl 5.92 to 19.63; Pyribenzamine 9.45 to 28.48 (see column 7, Tables I and II). The composite slopes for each pair of dosage-mortality curves are shown in column 11 of Tables I and II. For sodium phenobarbital and sodium pentobarbital the range was from 13.82 to 27.72; for Benadryl and Pyribenzamine, 9.32 to 19.56.

Unfortunately, some of the collaborators did not inject the drugs simultaneously and this meant that all their data could not be used in formulating our conclusions. Of those who were able to follow the directions, it is of interest to note that in only one case, laboratory No. 8, Table II, did the slopes of the dosage-mortality curves within laboratories differ significantly from one another. This fact may be observed by an inspection of the χb^2 values in column 8, Tables I and II. This would indicate that the two drugs which were injected simultaneously were qualitatively alike in their action on mice.

One of the objectives of this study was to find out what the differences in LD_{50}'s would be between laboratories for each of these drugs when the experimental conditions were about the same. The LD_{50} estimates are given in column 9, Tables I and II, and their respective limits of error are given in column 10, Tables I and II. For sodium phenobarbital the range was 205–279 mg./Kg.; sodium pentobarbital 122–137 mg./Kg.; Benadryl 74–117 mg./Kg.; Pyribenzamine 57–85 mg./Kg. The differences between some of these values are not significant but the extreme values in each case are real differences. Expressed as ratios, the following values are obtained: sodium phenobarbital 1.36; sodium pentobarbital 1.12; Benadryl 1.58; and Pyribenzamine 1.49.

For those collaborators who were able to carry out the simultaneous injection of each pair of drugs as directed, the observed toxicities in column 12 Tables I and II, may be compared. In each case the toxicity of sodium pentobarbital is expressed as a percentage of sodium phenobarbital, and also the toxicity of Pyribenzamine is expressed as a percentage of Benadryl. For sodium phenobarbital and sodium pentobarbital comparisons, only five values may be considered and the range for these is from 178 to 201. Four of these results are in very close agreement, 201, 200, 201, and 194, respectively. For the Benadryl and Pyribenzamine comparisons, only six results may be considered, due to the fact that two collaborators did not carry out the tests simultaneously. These six laboratories reported toxicities of 137, 144, 141, 120, 140, and 158, respectively.

A *chi*-square test (4) was applied to these estimates, and five of the six Benadryl-Pyribenzamine values were found to agree. The estimate for laboratory No. 4 differed from the others. Of the five comparisons on the barbiturates, four agreed. The reason for these two laboratories differing from the others has not been ascertained. It is possible it may have been due to some slight unrecognized modification in technique for one of the drugs, or differences of this magnitude may have been expected from normal sampling variation.

Even including those estimates which were slightly at variance with the majority of the others, the differences between these estimates are less than those obtained by comparing the ratios of extreme LD_{50} values between laboratories for the respective drugs. To illustrate this point further, the following example is presented: if we were presented with a table of LD_{50} values obtained from several laboratories at different times for these drugs, we would expect to have a series of values similar to those listed in column 9 of Tables I and II. If we wanted to know the relative toxicity of one of these compounds in terms of the other, we would very likely compare their LD_{50} values. We would have a series of ratios as follows: e. g., for sodium phenobarbital and sodium pentobarbital these ratios would range from 1.50 to 2.29; for Benadryl and Pyribenzamine, 0.87 to 2.06. Having obtained these ratios we would have to decide which is the correct one.

To overcome this difficulty, we would suggest that a more satisfactory method of evaluating the toxicity of similar-acting drugs would be to simultaneously inject the drugs under study, according to a definite procedure, and calculate the relative toxicities as has been done with these data, or use some similar method.

We do not wish to imply that we are opposed to the method of reporting the acute toxicity of a drug as the LD_{50} in milligrams/cc. per kilogram of body weight. This method is useful for estimating the approximate toxicity of a drug. However, in many instances the main objective of the investigator is to find out the toxicity of a drug in terms of another similar-acting drug of established use. For this purpose we feel the method we have followed, or a similar one, is to be preferred. One additional point might be mentioned in this connection, and that is, where the slopes of the two drugs being tested consistently prove significantly different, another similar-acting drug with a slope approaching that of the unknown would be a more suitable standard against which to test the unknown, because the significant difference in slopes would indicate that the drugs do not act alike, as originally anticipated.

This method is not only applicable where the tests are conducted simultaneously under similar testing conditions, but also where it is desirable to vary the experimental conditions for one of the drugs under study.

CONCLUSIONS

As far as our main objectives in this study are concerned, we have shown that even when these drugs are tested under conditions as nearly identical as possible, the LD_{50} values vary significantly between laboratories, but it has been possible to estimate the relative toxicities for the drugs selected, provided the drugs were tested simultaneously. It would also appear that this method of determining the comparative toxicity of drugs is a more reliable index of toxicity than a comparison of LD_{50} values obtained at different times within the same laboratory, or from different laboratories.

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Water-Soluble Embedding Materials for Botanical Microtechnique*,†

By ROBERT L. VAN HORNE‡ and LOUIS C. ZOPF§

A method employing water-soluble polyethylene glycols and solid materials of the same type of structure called "Carbowaxes" has been developed for the preparation of slides of plant materials. The specimens are killed and fixed in the usual manner and are then run up in dilutions of polyethylene glycol until they are completely dehydrated in 100 per cent glycol and are then embedded in the solid "Carbowax" matrix. Sectioning and mounting of the sections on glass slides is described and staining techniques are explained.

A METHOD employing water-soluble polyethylene glycols and solid materials of the same type of structure called "Carbowaxes" has been developed for the preparation of slides of plant materials.

The specimens are killed and fixed in the usual manner and are then run up in dilutions of polyethylene glycol and water until they are dehydrated in pure polyethylene glycol. Embedding with a "Carbowax" mixture follows. Sectioning and mounting of the sections on slides is described and staining techniques are explained.

Among the plant materials so treated were representative tissues of the leaves and stems of *Nicotiana*, *Ficus*, *Digitalis*, and flowers of *Yucca*.

The results and merits of the method are described and evaluated. Photomicrographs of sections were prepared and are presented to illustrate the results of the method.

Many papers have appeared in recent years suggesting the use of the water-soluble substances known as the "Carbowaxes"[1] (1) as the matrix for sectioning animal tissues (2–5). There are several reasons which indicate the selection of "Carbowax" instead of paraffin as the inclusion medium. They are:

1. The time necessary for dehydration preceding the paraffin embedding is shortened by the new method.
2. The proposed dehydrating procedure eliminates much of the shrinkage of tissues which follows the use of alcoholic dehydrating fluids in the paraffin process.
3. "Carbowax" may be obtained in varying degrees of hardness and the melting point of the preparation can be varied as desired.

"Carbowaxes" have been infiltrated into eye tissues in a matter of four days (3) while the same process with paraffin requires up to three and one-half weeks. In addition, since the "Carbowaxes" require no previous dehydration of the animal tissue with alcohol before the infiltration process, some of the shrinkage of the tissue is eliminated.

The application of the "Carbowaxes" to the botanical field of microtechnique should present the same advantages as have been achieved in their use in animal histological work. Paraffin,

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[1] Carbide and Carbon Chemicals Corporation, New York.

celloidin, and other embedding agents commonly used in plant microtechnique, require the prior dehydration of the plant material with alcohol or other organic solvents, with resultant plasmolysis or shrinkage of cells. This is due, apparently, to the removal of bound water from the cells along with the free water. Johansen (6) recommended the adoption of a technique which would give satisfactory dehydration without the harsh effects of the normal dehydrating agents.

The liquid, lower-molecular-weight, forms of the "Carbowaxes" known as the polyethylene glycols suggest themselves as desirable dehydrating agents since they have a strong affinity for water. They are available in varying degrees of viscosity, depending upon their molecular weights, which range from 200 to 600 (7). Also, they have the advantage of being completely water-soluble and are miscible with most organic solvents, with the exception of some of the aliphatic ethers and hydrocarbons. Studies on their toxicity when applied to the skin and when ingested (8, 9) show them to be innocuous to animals.

EXPERIMENTAL

The object of this investigation was to determine a routine method for the preparation of microscopic slides of plant materials using polyethylene glycols as the dehydrating agents and the "Carbowaxes" as the matrix for embedding and sectioning. The method eliminates, to a great extent, the shrinkage and plasmolysis of the tissues which occur when the paraffin method is used.

As mentioned in Carsten's paper (3), the addition of a small percentage of a surface-active agent to the "Carbowax" accelerates its infiltration into the animal cells. The use of about 1 to 2% of Tween 20[1] (10) was adopted in the "Carbowax" mixture for embedding the plant specimens. Tween 20 is a water-soluble, nonionic, liquid wetting agent. It was not added to the dehydrating solutions of polyethylene glycol as it did not appear to shorten materially the time necessary for dehydration.

Dehydration.—The technique used by microtechnicians in which they infiltrated the animal tissues directly with melted "Carbowax" without prior dehydration was unsatisfactory for plant histological work. When plant specimens were immersed directly in melted "Carbowax," they shrank grossly and were much distorted. It was found, however, that by dehydrating the specimens in a series of polyethylene glycol dilutions with water, ending in pure, undiluted polyethylene glycol, no distortion of the plant tissues resulted upon infiltration with a mixture of "Carbowaxes."

The solutions were as follows:

1. 25% polyethylene glycol 400W, 75% water
2. 50% polyethylene glycol 400W, 50% water
3. 80% polyethylene glycol 400W, 20% water
4. 95% polyethylene glycol 400W, 5% water
5. 100% polyethylene glycol 400W.

The plant specimens were killed and fixed in F. A. A., or other suitable killing fluid, and were washed in water before starting the dehydration process. The first solution was poured on the specimens, contained in a shell vial, and at least one-half hour was allowed for the penetration of the fluid into leafy specimens, or a suitable longer time for dense or woody tissue. An indication of the time needed for complete penetration of the solution was determined by watching for the tissue to settle to the bottom of the vial. After the first liquid had infiltrated the tissue, it was replaced by the second solution and the process continued until several changes of 100% polyethylene glycol 400W assured complete dehydration.

Embedding.—The embedding mixture—consisting of "Carbowax" 4,000 15 to 25%, Tween 20 1 to 2%; and "Carbowax" 1,540 as the remainder—was melted and transferred to a vial, where it was allowed to congeal without hardening. The dehydrated specimen was placed on top of the mixture in the vial and the vial transferred to a warming oven where the wax melted slowly, allowing the specimen to settle to the bottom. Following this, the wax was replaced with a fresh portion of melted "Carbowax" mixture and the process was finally repeated once more, so that the vial remained in the oven at least twelve hours. A change of the melted "Carbowax" mixture was made just before embedding the specimen. This period of time in the melted wax sufficed for all but the more dense or woody tissues. Such tissues usually required several hours more in the oven to insure thorough penetration of the "Carbowax" into the specimens.

Figure 1 is a curve showing the melting range of mixtures of "Carbowax" in various proportions. As the percentage of "Carbowax" 4,000 was increased, the melting range rose, as did the hardness of the mixture.

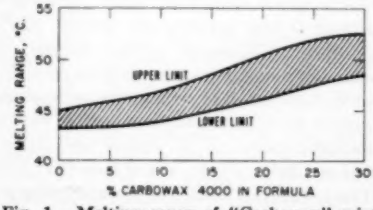

Fig. 1.—Melting range of "Carbowax" mixture with varying percentages of "Carbowax" 4000. Melting range: "Carbowax" 1540–43–45° C., "Carbowax" 4000–52–55° C.

The procedure for casting the blocks of "Carbowax" mixture containing the embedded material was the same as that used for paraffin. Rapid cooling of the melted mixture was necessary to insure fine crystalline structure and transparency of the block. This may best be done in a refrigerator, after which the blocks may be stored in closed glass jars to protect the "Carbowax" mixture from contact with moisture. Figure 2 shows that a relatively small amount of moisture is absorbed by a block of "Carbowax" mixture exposed to the atmosphere.

[1] Atlas Powder Company, Wilmington, Del.

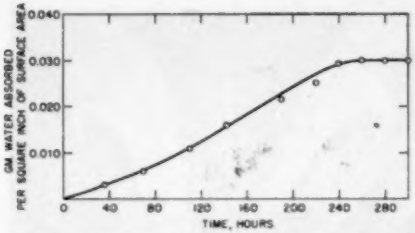

Fig. 2.—Hygroscopicity curve of mixture of "Carbowax" 1540–84%; "Carbowax" 4000–15%, Tween 20–1%. Room temperature–72° F., relative humidity–60%.

Sectioning.—The specimens were cut out of the block and mounted on suitable holders in the same manner as with paraffin. The only precaution which had to be observed was to avoid contact with water during the sectioning process.

Sectioning was done with the block at a temperature of about 10–15°, although reasonably good ribbons were cut even at room temperature. The knife and wax were cooled during the sectioning by allowing carbon dioxide gas from a tank to flow down over the blade and block (11). Sections have been cut at thicknesses of from 8 to 25 μ with good results. The ribbons are easily handled if due care is exercised to avoid contact with water.

Mounting.—The solubility of "Carbowax" in water and most other solvents posed a problem when the mounting of the sections on slides was undertaken. In order to obtain the desired flattening of the tissue, and also to cause the sections to adhere securely to the slides, two methods were developed. The first is applicable to most plant tissues which are not delicate or apt to tear readily when floated out on the slides.

Method One.—Drop a little Haupt's adhesive[3] on a slide and add two or three drops of 5% formaldehyde solution. Spread out the liquids with the finger to form a thin film, and at once lay out the ribbon of "Carbowax" mixture on the liquids. Place the slide on a warming plate at 42° and dry the slide for eight hours or more. This method produces adequate expansion of the ribbon without dissolving the "Carbowax" mixture. The wax then may be removed from the sections by dipping the slide into water, and then the usual staining procedures may be followed.

For very delicate tissues and for those which do not adhere well to the slide after the foregoing process, the following method may be employed as the flotation method.

Method Two.—Smear a little Haupt's adhesive on the slide and flood it with normal butyl ether. This ether does not dissolve "Carbowaxes" and causes little brittleness in the wax. Place the ribbon on the ether and warm the slide on the warming plate as before until the desired flattening has occurred. Remove the excess ether by blotting it carefully from the edge of the slide and allow the slide to dry. The "Carbowax" mixture is removed by dipping the slide into 100% ethyl alcohol. After ten minutes, pass the slide into a 1% solution of celloidin in equal parts of absolute ethyl alcohol and anhydrous ethyl ether. About five minutes in this solution is adequate. Remove the slide and allow it to remain in the air until the celloidin is nearly dry, at which time the slide is immersed in 95% ethyl alcohol to harden the celloidin. Henceforth, the standard procedure for passing the slide down the alcohol series into the desired stain is followed.

Staining.—Rapid safranin and fast green were used to stain the plant materials prepared by this method. Among the specimens employed were *Helianthus* stem, *Eucalyptus* stem, *Anise hyssop* leaf and stem, *Digitalis* leaf, *Yucca* flower, *Ficus* leaf, and *Nicotiana* leaf and stem. By using the harder "Carbowax" mixture containing 25% of "Carbowax" 4,000, no difficulty was experienced in cutting good sections of the woody stem.

Figure 3 is a section through the leaf of *Anise hyssop* cut in "Carbowax" mixture. *Nicotiana* leaf also sections reasonably well, as is shown by Fig. 4. Figure 5 is a section through the ovule of

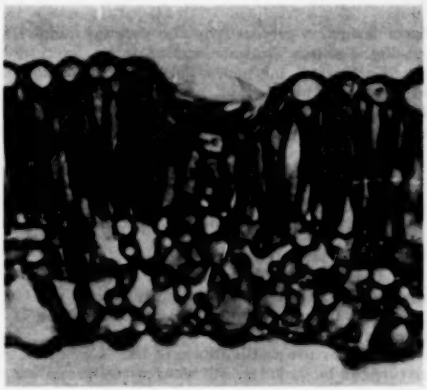

Fig. 3.—Section through the leaf of *Anise hyssop* embedded and cut in "Carbowax."

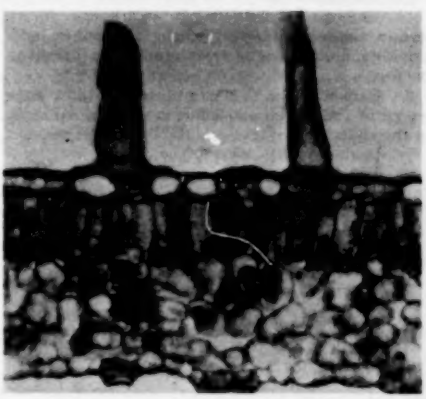

Fig. 4.—*Nicotiana* leaf prepared by the "Carbowax" technique.

[3] Haupt's adhesive: 1% gelatin in distilled water, with 2% phenol and 15% glycerin added.

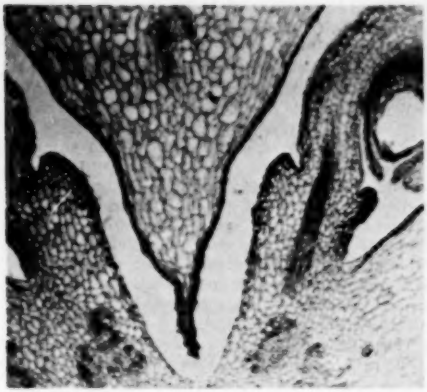

Fig. 5.—*Yucca* flower run up in paraffin. Shrinkage of the tissue is evident.

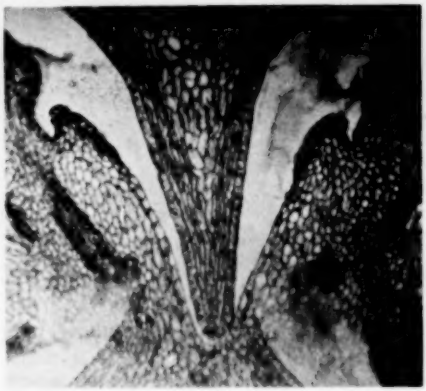

Fig. 6.—Another section of *Yucca* flower prepared by the "Carbowax" method. Note the lack of distortion of the cells lining the cavity in this section.

Yucca flower as prepared by the paraffin method, and Fig. 6 shows the same section as prepared by the "Carbowax" method. The difference in the shrinkage and distortion resulting from use of alcoholic dehydration agents as compared with the polyethylene dehydrating fluids is evident.

DISCUSSION

Advantages of the "Carbowax" method are:

1. It is relatively cheap.
2. It is readily soluble in water and can be removed from both tissues and utensils easily.
3. The time for infiltration and embedding is somewhat shortened.
4. The texture and hardness of the "Carbowax" mixture can be varied at will along with the melting range.
5. The lack of distortion and shrinkage in the plant tissues is most desirable.

Certain disadvantages are also evident:

1. The water-solubility of "Carbowaxes" at certain times of the year when temperature and humidity are high is often a problem in handling the wax.
2. Care must be taken to insure adequate flattening of the ribbon and adherence of the sections to the slide.
3. Some tissues may require the use of celloidin to fix them on the slide when delicate or easily disrupted.

SUMMARY

A method for the preparation of slides of plant materials using water-soluble polyethylene glycols and "Carbowaxes" for dehydration and embedding has been proposed. Good results were obtained with nearly all of the specimens prepared by this method.

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A Simple Quantitative Method for the Determination of Progesterone in Oil Solution*

By HERMAN COHEN and ROBERT W. BATES

A procedure is described for the quantitative gravimetric determination of progesterone, utilizing a stable solution of the carbonyl reagent 2,4-dinitrophenylhydrazine. This method is applicable directly to solutions of progesterone in oil without extraction.

ELVIDGE (2) has reviewed the existing chemical methods for the determination of progesterone and has discussed their inadequacies. Recently, Klein, *et al.* (3), described a method utilizing the carbonyl reagent 2,4-dinitrophenylhydrazine (1) for the quantitative determination of progesterone. A similar quantitative method for estrone was described in a paper by Veitch and Milone (4).

The method presented here is a modification of the method of Klein, *et al.* It utilizes a reagent solution which is stable, and thus eliminates individual weighings of the reagent for each sample. The heating time is reduced to about one minute, which eliminates refluxing, and the reaction is performed directly on oil solutions, thereby eliminating extractions.

METHOD

Apparatus.—25-ml. Erlenmeyer flasks; filter crucibles of 20–30 ml. capacity, medium porosity.

Reagents.—2,4-Dinitrophenylhydrazine (Eastman); HCl C. P.; absolute ethanol; petroleum ether.

Preparation of Reagent.—The 2,4-dinitrophenylhydrazine reagent solution is prepared as follows: to 500 mg. of 2,4-dinitrophenylhydrazine is added 5 ml. of HCl C. P. and the mixture shaken until all of the 2,4-dinitrophenylhydrazine has turned canary yellow, indicating conversion to the hydrochloride. One hundred milliliters of absolute ethanol is added and the mixture is heated until the 2,4-dinitrophenylhydrazine hydrochloride is dissolved. One milliliter more of HCl C. P. is then added. The solution is allowed to cool and is placed in a refrigerator overnight. The small white precipitate which usually forms is then filtered off and the solution is ready for use.

This reagent solution contains 5 mg. of 2,4-dinitrophenylhydrazine per ml. and is stable for at least three months when kept in the refrigerator.

Crystalline Progesterone.—To an absolute ethanol solution of progesterone (5–10 mg./ml.) in a 25-ml. Erlenmeyer flask is added sufficient reagent so that there will be 3 mg. of 2,4-dinitrophenylhydrazine for each mg. of progesterone. The mixture is brought to a boil on a steam bath for a period of about one minute. It is then removed and allowed to remain at room temperature for one hour. The precipitate is filtered off, washed twice with ethanol containing 6% HCl (1 ml. HCl C. P. plus 5 ml. ethanol) to remove any unreacted 2,4-dinitrophenylhydrazine, and washed finally with 5 ml. of H_2O. The precipitate is dried at 110° to constant weight. Weight of the derivative $\times$ 0.466 = weight of progesterone in the sample. The derivative is orange-red in color and melts at 272–274° (Fisher-Johns apparatus, uncorrected). The structure of the derivative is discussed by Klein, *et al.* (3). It is insoluble in petroleum ether, acid-alcohol, and water. Results obtained using this procedure are presented in Table I.

TABLE I.—ANALYSES OF ETHANOL SOLUTIONS OF CRYSTALLINE PROGESTERONE

Progesterone in Sample, Mg.	Weight of Hydrazone, Mg.	Progesterone Found, Mg.	Progesterone Found, %
9.92	20.9	9.74	98.1
9.92	21.2	9.88	99.6
9.92	20.9	9.74	98.1
9.92	21.0	9.79	98.7
9.92	20.8	9.69	97.7
9.92	20.8	9.69	97.7
9.92	20.8	9.69	97.7
10.00	21.3	9.93	99.3
10.00	21.3	9.93	99.3
10.00	21.3	9.93	99.3

Progesterone in Oil.—To a 1-ml. sample of oil solution of progesterone (2–25 mg. of progesterone per ml.) in a 25-ml. Erlenmeyer flask are added 5 to 10 volumes of petroleum ether or hexane and sufficient hydrazine reagent so that there will be 3 mg. of 2,4-dinitrophenylhydrazine for each milligram of progesterone. After the addition of the hydrazine reagent, two phases are present, but this does not interfere with the course of the reaction. This mixture is brought to a boil for about one minute on a water bath and then placed in the refrigerator for one hour. At the end of this period of time, the precipitate is filtered off and washed several times with petroleum ether (to remove any oil which may be occluded in the precipitate), followed by two washes with 6% HCl in ethanol. The precipitate is dried to constant weight at 110°.

Corn oil and sesame oil similarly treated gave no precipitate.

Results obtained using this procedure upon samples of progesterone in oil that were prepared in our laboratory are presented in Table II.

Table III is a presentation of results obtained using this procedure to determine the progesterone

* Received June 7, 1950, from the Squibb Institute for Medical Research, New Brunswick, N. J.

TABLE II.—ANALYSES OF PREPARATIONS OF CRYSTALLINE PROGESTERONE IN CORN OIL

Progesterone in Sample, Mg.	Weight of Hydrazone, Mg.	Progesterone Found, Mg.	Progesterone Found, %
5.00[a]	10.3	4.80	96.0
5.00[a]	10.4	4.85	97.0
5.00[a]	10.4	4.85	97.0
10.00[a]	20.4	9.51	95.1
10.00[a]	21.1	9.83	98.3
10.00[a]	20.4	9.51	95.1
10.70[b]	22.3	10.39	97.1
10.70[b]	22.9	10.67	99.7
10.70[b]	23.4	10.90	101.9
10.08[b]	22.3	10.39	103.1
10.08[b]	21.9	10.21	101.3
10.08[b]	21.4	9.97	98.9
10.08[b]	21.7	10.11	100.3
15.00[a]	31.2	14.54	96.9
15.00[a]	32.6	15.19	101.3
15.00[a]	32.3	15.05	100.3

[a] Sample in 1 ml. of oil.
[b] Sample in 0.5 ml. of oil.

content of commercial preparations of progesterone in oil, containing 5% in excess of the labeled potency.

The satisfactory recoveries obtained with this method upon both laboratory and commercially prepared samples of progesterone in oil indicate its adequacy and usefulness in control procedures.

TABLE III.—ANALYSES OF COMMERCIAL SAMPLES OF PROGESTERONE IN CORN OIL

(All Samples Were in 1 Ml. of Oil Containing 2% Benzyl Alcohol)

Progesterone[a] in Sample Mg.	Weight of Hydrazone, Mg.	Progesterone Found, Mg.	Progesterone Found, %
2.1	4.5	2.1	100.0
2.1	4.4	2.05	97.6
2.1	4.3	2.0	95.2
2.1	4.5	2.1	100.0
5.25	11.5	5.36	102.0
5.25	12.0	5.59	106.5
5.25	11.5	5.36	102.0
10.5	22.1	10.30	98.1
10.5	21.9	10.21	97.2
10.5	21.9	10.21	97.2
26.25	54.2	25.26	96.2
26.25	54.2	25.26	96.2
26.25	53.8	25.07	95.5

[a] Includes the 5% excess above potency on label.

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Studies on *Mahonia* Genus. III. *M. borealis* Takeda and *M. Simonsii* Takeda*

By R. CHATTERJEE, M. P. GUHA, and S. K. SEN†

From the roots of *M. borealis* Takeda and *M. Simonsii* Takeda have been isolated the alkaloids, oxyacanthine, berberine, neprotine, palmatine, and jatrorrhizine, in different quantities. The subtle morphological differences of several species of *Mahonia* which were listed once as *M. nepalensis*, e. g., *M. acanthifolia* Don, *M. nepalensis* D. C., *M. philippinensis* Takeda, and of *M. acanthifolia* and *M. Simonsii*, have been justified with reference to the difference in quantity and nature of their alkaloids. *M. manipurensis* Takeda has been called a variety of *M. borealis* Takeda in preference to a true species.

IT HAS been found that three classes of chemical compounds, alkaloids, glycosides, and volatile oils, are aids to the classification of plants. In the genus *Aconitum* for instance, two Indian species were formerly called *A. ferox* which were later separated to *A. deinorrhizum* and *A. spicatum*, and it was found that the former species contained the base pseudoaconitine $C_{34}H_{51}O_{12}N$, and the latter, bikhaconitine, $C_{34}H_{51}O_{11}N.H_2O$. Similarly, the name of some of the Japanese species which were called *A. Fischeri* was finally settled by the isolation of japaconitine, $C_{34}H_{49}O_{11}N$ from an authentic specimen of *A. Fischeri*, and jesaconitine, $C_{36}H_{51}O_{12}N$ from a different species, which was then called *A. japonicum* (1).

It was known that *Lycopodium clavatum* grew in Europe and America. The European *clavatum* yielded three alkaloids, lycopodine, clavatine, and clavatoxine. It had been found the Canadian *clavatum* yielded only lycopodine. This, therefore, called for the revision of the nomenclature of these plants growing in two different places, for the varietal distinction or habitat should have a profound influence upon the nature of the elaborated alkaloids (2).

* Received May 15, 1950, from the Department of Chemistry, Presidency College, Calcutta, India.
† The authors are indebted to Dr. P. C. Rakshit, Head of the Department of Chemistry, Presidency College, for providing them with laboratory facilities.

The identification of plants exclusively with the help of isolated alkaloids is not without objection. When attempts are made to reclassify genera, and species within a genus, the chemical results are not so concise. This is obviously due, in part, to the limited number of plants examined and to the fact that the constitution of many of the alkaloids has not yet been determined. As has been seen before, some conclusions can, nevertheless, be drawn. The chemical examination of plants has one point in its favor: it does not depend upon preconceived ideas of classification so that when taxonomists and chemists fail to agree, the latter have the advantage. In the study of *Mahonia* genus (3), it was found that *M. acanthifolia* Don was recognized as a species distinct from *M. nepalensis* of De Condolle. Later botanists thought only one species of *Mahonia*, *M. nepalensis* D. C., was known to be a native of India and this species was supposed to be distributed over the whole range of the Himalayas, Nepal, Assam, and even to the Philippines. Takeda (4), however, differentiated *M. nepalensis* from *M. philippinensis*, and also declared *M. acanthifolia* as a separate species. It has now been found that this classification is substantiated by the separation of different alkaloids from these species (Table I).

TABLE I.—THE ALKALOIDS OF DIFFERENT SPECIES OF *Mahonia*

M. nepalensis (5)	*M. acanthifolia* (7)	*M. philippinensis* (6)
Umbellatine	Oxyacanthine	Berberine
Neprotine	Berberine	Shobakunine
	Neprotine	Jatrorrhizine
	Palmatine	
	Jatrorrhizine	

M. manipurensis Takeda is considered to be distinct from *M. borealis* Takeda, but their morphological differences (as will be evident from Table II) are more apparent than real. The flowers of *M. manipurensis* appear to be similar to those of *M. borealis*. The similarity of these two species appears to be more striking when it is found by the present writers that in both cases the per cent of berberine is slightly less than that of neprotine (details to be published in part IV of this series). A comparative histology of the different tissues also points to the close similarity of the two species. It seems well, therefore, to consider *M. manipurensis* as a variety of *borealis* rather than a separate species.

On the other hand, though closely similar in external appearance and production of the same five alkaloids, the species *M. acanthifolia* Don and *M. Simonsii* Takeda should be considered to be different since the relative proportion of these alkaloids in them differs widely.

TABLE II.—THE POINTS OF DIFFERENCE OF *M. borealis* TAKEDA AND *M. manipurensis* TAKEDA

	M. borealis	*M. manipurensis*
Pedicels	Rather thick, shorter than bracts	Filiform, either equal to, or longer than, bracts
Connective of stamens	Triangular, apiculate	Triangular, depressed
Style of the berry	1 mm. long, ornate	Short

TABLE III.—THE PER CENT OF ALKALOIDS IN THE MORPHOLOGICALLY ALLIED SPECIES

	M. acanthifolia	*M. Simonsii*	*M. borealis*
Oxyacanthine	Traces	Traces	Traces
Berberine	0.5	0.021	0.16
Neprotine	0.0068	0.27	0.311
Palmatine	0.07	0.024	0.031
Jatrorrhizine	0.138	0.02	0.038
Approx. ratio of berberine to neprotine	70:1	1:13	1:2

EXPERIMENTAL

The plant materials, the roots of *M. borealis*, were obtained through Mr. Raizada, the Forest Botanist, Dehra Dun, and the roots of *M. Simonsii* were collected by Dr. S. K. Mukerjee, Curator of the Herbarium, Indian Botanic Gardens, Calcutta, during his botanical expedition to Manipur State in 1947–1948.

Air-dried, finely powdered (60 mesh) root (1 Kg.) of each species was extracted exhaustively with warm ethanol (95%). The solvent was removed under reduced pressure and the sticky brown residue was treated with iced water (300 cc.) and filtered three times to remove resinous matter. The clear dark-brown liquid was cooled to 0° and made alkaline with ammonia (25%), when a sticky amorphous solid separated, and the mixture was extracted five times with ether.

Treatment of the Ethereal Layer

The yellow-colored ethereal solution was washed twice with water, and, on removal of ether, a sticky resinous residue (about 1 Gm.) was left behind. This was Soxhleted with dry ether (50 cc.) and most of it dissolved. The solution was filtered; kept for three days in a refrigerator, during which time a very small quantity of practically colorless crystals separated. These crystals were treated with the least quantity of dilute hydrochloric acid (5%); filtered from insoluble matter; and kept in the refrigerator until crystals of a hydrochloride separated.

The Crystals: Identification of Oxyacanthine.—The pale yellow crude hydrochloride of the base was dissolved in warm water and was treated with ammonia to precipitate an amorphous base, which

was taken up in ether and the ethereal solution, on long standing in the refrigerator, yielded colorless crystals (in traces), which on recrystallization from methanol melted at 216° *in vacuo* and did not depress the melting point of an authentic sample. On adding a drop of ferric chloride solution, followed by potassium ferricyanide, to the base, a green coloration was obtained which rapidly changed to blue, the characteristic color reaction of oxyacanthine.

Both the species, *M. borealis* and *M. Simonsii*, yielded this base in traces.

The Mother Liquor.—The mother liquor could not be worked up successfully in either of the above two cases.

Treatment of the Aqueous Layer

In the Case of *M. borealis*.—The clear brown aqueous solution (400 cc.) was treated with hydrochloric acid to give a 1% acid solution and was allowed to stand overnight in the refrigerator, but no solid product was obtained. The solution was then left in a large crystallizing dish for slow evaporation at room temperature. After about ten days, a dark crystalline solid mixed with much tarry impurities deposited, and the mother liquor was carefully filtered off.

Treatment of the Crystals: Separation of Berberinium Chloride from Neprotine Hydrochloride

The crude product (6.6 Gm.), on repeated washing with small quantities of dilute ethanol, became bright orange-yellow in appearance and was subjected to the process of fractional crystallization. In this way the following fractions could be separated:

(*a*) The first fraction: pure yellow crystals, 0.94 Gm.; (*b*) The second fraction: mixture of yellow and red crystals, 2.70 Gm.; (*c*) The third fraction: pure red crystals, 1.7876 Gm.; (*d*) The mother liquor.

Treatment of the First Fraction (Pure Yellow Crystals)—*Identification of Berberine.*—The yellow hydrochloride furnished lemon yellow acetone compound, m. p. 166–168° dec., which did not depress the melting point of an authentic specimen of anhydroberberineacetone, 167–168° dec. The derivative obtained on reduction with zinc dust and glacial acetic acid melted at 172–173° and did not depress the melting point of an authentic sample of tetrahydroanhydroberberine. The free base obtained from the above alkaloid was further purified by repeatedly crystallizing from dilute ethanol, and then converted into the chloroplatinate salt in the usual manner, which on analysis furnished the following results. Found: Pt, 18.03 [$(C_{20}H_{17}O_4N)_2 \cdot H_2PtCl_6$)], requires Pt, 18.02%.

Treatment of the Second Fraction: Separation of Berberine and Neprotine by the Acetone Method.—The second fraction (2.7 Gm.) consisted of a mixture of berberinium chloride and neprotine hydrochloride and could not be further separated by the method of fractional crystallization. These were, therefore, subsequently separated more successfully by the acetone method, previously suggested by Chatterjee and Guha in the case of *M. acanthifolia* (7).

A solution of the mixture of the hydrochlorides in water (200 cc.) was warmed up to 50°, made alkaline with 10% sodium hydroxide solution (40 cc.), treated with acetone (100 cc.), the mixture was allowed to cool, and shining lemon yellow crystals separated. From the well-cooled mixture the crystals of anhydroberberineacetone (0.47 Gm.), m. p. 166–168° dec., were removed by filtration and the filtrate was acidified in the cold by the least quantity of concentrated hydrochloric acid, added dropwise. The acidified solution, on slow evaporation at room temperature, deposited shining red needles (1.3108 Gm.) after a week. The product was identified as neprotine hydrochloride, since it furnished a picrate, m. p. 208–210° dec., which did not depress the melting point of an authentic sample prepared from *M. nepalensis* (5, 7).

Treatment of the Third Fraction (Pure Red Crystals)—*Identification of Neprotine.*—The red hydrochloride charred without melting and furnished an orange picrate, m. p. 208–210° dec., which did not depress the melting point of an authentic sample of neprotine picrate.

Treatment of the Mother Liquor—*Isolation of Neprotine Picrate.*—The mother liquor remaining after the separation of the last fraction of neprotine hydrochloride was further diluted by adding ethanol, and a dilute solution of picric acid was added to the warm solution until the solution suddenly became pale. It was allowed to cool, and as the solution cooled, beautiful shining orange needles (0.44 Gm.) gradually separated. These were, however, found to be identical with neprotine picrate.

In this way it was finally possible to account for the following yields of the above two alkaloids from the dried root of *M. borealis:* berberine, 0.16%; neprotine, 0.311%.

Treatment of the Crystals in the Case of *M. Simonsii*.—The crystals of the mixture of hydrochlorides obtained from this species were similarly worked up and ultimately the two alkaloids, berberinium chloride and neprotine hydrochloride, were isolated in the following proportions: berberinium chloride, 0.22 Gm., hence berberine, 0.021%; neprotine hydrochloride, 2.91 Gm., hence neprotine, 0.27%.

Treatment of the Filtrate

Isolation of the Iodides in the Case of *M. borealis*.—The mother liquor obtained after the separation of the hydrochlorides was treated with sodium acetate (10 Gm.), followed by a solution of potassium iodide (10 Gm.) in water, and a brown iodide immediately separated. It was removed by filtration and the filtrate left in the refrigerator overnight for further crystallization. A second crop of golden yellow iodide slowly separated from the filtrate. First crop of crystals: brown iodide, 6.64 Gm.; second crop of crystals: golden yellow iodide, 0.40 Gm.

Isolation of the Iodides in the Case of *M. Simonsii*.—The mother liquor in this case also was treated as described under *M. borealis* but the dark brown iodide which initially separated had a tendency to become sticky on the filter paper when attempts were made to filter the product. The solid was, therefore, carefully rinsed with ethanol, and the sticky matter dissolved in the solvent; the iodide

became brighter and granular and could be easily filtered out. The residue, on drying in a vacuum desiccator, was finally obtained as a brown powdery mass. The second crop of golden yellow iodide was, however, obtained as in the previous case. First crop of crystals: brown iodide, 5.0 Gm.; second crop of crystals: golden yellow iodide, 0.20 Gm.

Treatment of the First Crop of Crystals (Brown Iodide)

Isolation of Tetrahydropalmatine and Tetrahydrojatrorrhizine in the Case of *M. borealis*.—The crude, dark brown iodide was purified by crystallization from ethanol and the pure yellowish-brown iodide (5.0 Gm.) was converted into the more soluble chloride by digesting with freshly precipitated silver chloride, according to the method outlined in the case of *M. acanthifolia* (7). The hot, dark brown solution obtained after filtration was immediately reduced by zinc dust and glacial acetic acid, and the reduced product separated into nonphenolic and phenolic portions as before. Reduced nonphenolic base, 0.3 Gm.; reduced phenolic base, 0.114 Gm.

Treatment of the Reduced Nonphenolic Base—*Identification of Tetrahydropalmatine.*—The product (0.3 Gm.) on crystallization from methanol melted at 142–143° and did not depress the melting point of an authentic sample of tetrahydropalmatine.

Treatment of the Reduced Phenolic Base—*Identification of Tetrahydrojatrorrhizine.*—The product (0.114 Gm.) on crystallization from methanol melted at 205–206° and did not depress the melting point of tetrahydrojatrorrhizine similarly obtained from *M. acanthifolia*.

Isolation of Tetrahydropalmatine and Tetrahydrojatrorrhizine in the Case of *M. Simonsii*.—The crude brown iodide (5.0 Gm.) was worked up in the same way as outlined above, and the following two products were finally isolated: tetrahydropalmatine, 0.23 Gm.; tetrahydrojatrorrhizine, 0.104 Gm.

Treatment of the Second Crop of Crystals (Golden Yellow Iodide)

Identification of Jatrorrhizine Iodide in the Case of *M. borealis*.—The product (0.4 Gm.) was dissolved in hot ethanol, treated with animal charcoal, and then filtered. The filtrate, on evaporation in a crystallizing dish, furnished golden yellow crystals (0.36 Gm.) melting at 210–212° dec., which did not depress the melting point of an authentic sample of jatrorrhizine iodide obtained from *M. acanthifolia*.

It therefore appears that under the present condition a fractional crystallization of the mixture of iodides has been achieved, thus leading to the separation of a small fraction of jatrorrhizine iodide in a practically pure state.

On the basis of the yields of the iodides, therefore, the above two alkaloids may be considered to be present in the following amounts: palmatine, 0.031%; jatrorrhizine, 0.038%.

Identification of Jatrorrhizine Iodide in the Case of *M. Simonsii*.—The second crop of iodide (0.2 Gm.) obtained in this case was similarly treated so as to yield golden yellow crystals (0.12 Gm.), which were identified with jatrorrhizine iodide, as stated above.

The yields of the two alkaloids, palmatine and jatrorrhizine, in this species have been found to be as follows: palmatine, 0.024%; jatrorrhizine, 0.02%.

SUMMARY

1. The advantage of the chemical examination of plants in determining their botanical classification has been recalled.

2. From the roots of *M. borealis* Takeda and *M. Simonsii* Takeda, the alkaloids, oxyacanthine, berberine, neprotine, palmatine, and jatrorrhizine have been isolated in different proportions.

3. Although *M. manipurensis* has been considered by Takeda to be morphologically distinct from *M. borealis*, it has been found on close observation that these differences are more apparent than real. The similarity of these two species appears to be more striking, for they synthesize the alkaloids in nearly the same relative proportion, and the internal structures of their tissues also have close similarities.

4. It seems well to consider *M. manipurensis* as a variety of *M. borealis*, rather than a separate species.

5. Although closely similar in external appearance and production of the same five alkaloids, the species *M. acanthifolia* Don and *M. Simonsii* Takeda should be considered to be different, since the relative proportion of these alkaloids is widely different in them.

6. The classification of several species of *Mahonia*, e. g., *M. acanthifolia* Don, *M. nepalensis* D. C., *M. philippinensis* Takeda, which were listed once as *M. nepalensis* D. C., has been justified with reference to the difference in quantity and nature of the alkaloids in them.

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Methods of Assay for Peroxidases and Polyphenolases and Their Application to Enzyme Isolation*,†

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Accurate and rapid methods for the quantitative analysis of peroxidases and polyphenolases are described which allow for mechanical deduction of nonenzymic oxidation effects commonly found in crude plant extracts. These methods were used in evaluating the successive steps for a method of partial isolation of the enzymes of two species of *Impatiens*.

REFERENCES to the several species of *Impatiens* have been found in medical literature for nearly one hundred years. Chief among its uses has been an attributed activity in curing the dermatitis resulting from contact with the plant *Rhus radicans* L. (1–9). Recent studies (10, 11) have indicated that phenol-oxidizing enzymes may inactivate the phenolic active principle of *Rhus radicans* L. Such enzymes are widely distributed in the plant kingdom (12–15). The frequent use of plant juices in treating Rhus dermatitis, of which McNair (16) lists 109, supported the hypothesis that phenol-oxidizing enzymes of plant origin might be effective in inactivating the principles responsible for poison ivy dermatitis. Pharmacological tests with the plant juice of *Impatiens* and the enzyme products obtained by the methods described in this paper have been previously reported (17).

The purpose of this investigation was twofold. The first goal was to find an accurate and rapid method of assay for peroxidase and polyphenolase activity which could be used to determine the effectiveness of the various common methods of enzyme isolation. The second purpose of the investigation was to obtain an enzyme of sufficient purity for studies in the pharmacological activity of peroxidases and polyphenolases, to determine the role they play in the reputed activity of the juice of species of *Impatiens* in the treatment of Rhus dermatitis.

The methods of analysis of enzymes of the polyphenolase type fall into three general classifications: colorimetric, manometric, and titrimetric. Due to the time required for the latter two methods, they did not lend themselves for practical use in this study. Willstätter and Stoll (18) were among the first to use the pyrogallol substrate color reaction for assaying peroxidases. Various modifications for peroxidase and polyphenolase determinations have been evolved (19–21). Those colorimetric methods most commonly used fail to take into consideration the part that nonenzymic oxidants play in affecting the assay, and thus have limited application when applied to crude plant extracts. A rapid colorimetric method was evolved to meet the requirements for the quantitation of peroxidases and polyphenolases in crude plant extracts.

No work has been done previously on the isolation of the enzymes of species of *Impatiens*.

EXPERIMENTAL

Methods of Assay.—The enzyme preparations used were prepared by the process of Graubard and Nelson (22). The final product was used for the polyphenolase tests (to be referred to as potato enzyme "B"). The products obtained prior to the kaolin adsorption were used for the peroxidase tests (to be referred to as potato enzyme "A"). It was found on four processes of isolation that the kaolin adsorption and elution removed the peroxidase activity, as evidenced by guaiac and benzidine tests, but the eluate contained oxidase activity, as indicated by the indophenol, catechol, and pyrogallol tests. The test for tyrosinase using tyrosine as the substrate was negative in the eluate. The more sensitive spectrophotometric test, to be described below, indicated the presence of small amounts of peroxidase in the final product. Since this was purported to be a method of isolating tyrosinase, the results were reported to Professor Nelson. His associate, Professor Dawson, indicated that in their opinion the loss in monophenolase activity was due to a modification of the original enzyme rather than the separation of different proteins. If this be true, kaolin is a very effective adsorbent to accomplish this modification. It is also worthy of note that the kaolin removed practically

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all of the peroxidase activity of this potato enzyme, a fact not noted by the authors of the original paper.

The Willstätter and Stoll (18) process for the determination of peroxidase activity and the modifications of Keilin and Mann (19) for polyphenolases were used as the basis for this investigation of methods of quantitation. In these processes a definite amount of an enzyme preparation is added to a definite amount of water containing a quantity of pyrogallol. This mixture is agitated in a bath of controlled temperature for a definite length of time. A portion of the oxidized pyrogallol is then added to a solution of sulfuric acid to halt enzyme action. The product is extracted with ether and brought to volume. This solution is examined for density of color and this color is compared to standard solutions of purpurogallin. Activity is expressed in equivalent quantities of this standard.

Initial tests were made using potato enzyme B testing for polyphenolase activity. Purpurogallin was synthesized by the method of Perkin and Steven (23), and routine spectrophotometric examination made of ether solutions of this compound for the determination of area of maximum absorption and extinction coefficient. The Beckman Quartz Spectrophotometer Model DU was used in all assays at 440 mμ. A number of tests were run varying substrate-to-enzyme quantity relationships, time of reaction, and agitation with an auxiliary stream of air. Results were calculated on the basis of the extinction coefficient of purpurogallin. It was not possible to obtain a direct relationship between amount of enzyme used and amount of calculated purpurogallin formed using the polyphenolase enzyme solution B.

The process was modified to exclude the direct comparison with purpurogallin, eliminate the ether extraction, and to recognize the effect of other oxidants on the substrate by using a blank solution of the inactivated enzyme. These modifications were first investigated as a method of determining peroxidase activity and later applied to the polyphenolase solution tested above. They were found to be applicable in this regard under restricted conditions of enzyme and substrate quantity relationships. The process included the modification of using buffered solutions suggested by Sumner and Gjessing (20).

The general process is described as follows, with the specific details of the process applicable to potato enzyme solution A (diluted 1:10 with water) shown parenthetically. A quantity of enzyme solution was added to a 100-cc. flask containing a quantity (20 cc.) of phosphate buffer of optimum *p*H (6.6) for the enzymes and brought to volume. This solution was placed in a water bath maintaining the optimal temperature (25°), and oxygenated by a controlled supply of air. Known amounts (40 mg.) of powdered substrate pyrogallol were weighed in small wide-mouthed vials, and at a noted time dropped into the reaction mixture. Just prior to the addition of the substrate, 0.8 cc. of 3% H_2O_2 was added to the reaction mixture. At a definite and predetermined time interval (ten minutes) a 25-cc. aliquot of the reaction mixture was removed and added to a 50-cc. volumetric flask containing a sufficient quantity (10 cc.) of 20% H_2SO_4 to halt enzyme action. This was brought to volume and examined in the spectrophotometer, using as the comparator solution a similar quantity of enzyme inactivated maximally by boiling for three minutes and treated exactly as outlined above for the active enzyme solution. This comparator solution automatically deducted the color of the enzyme solution and nonenzymic oxidation of the substrate.

It was found in hundreds of assays using this technique that the logarithm of the transmittance bore a straight line relationship to the amount of enzyme solution (Fig. 1). Thus, by using the color

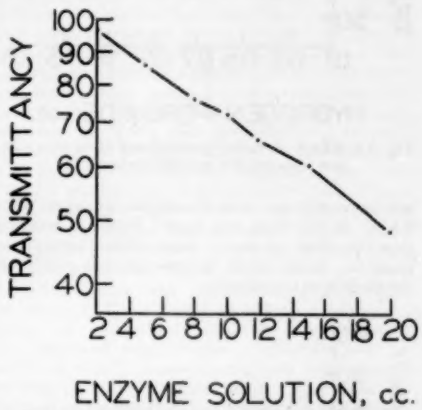

Fig. 1.—Straight line relationship between quantity of peroxidase and logarithm of transmittancy using the proposed method of assay.

of the total end products of oxidation of pyrogallol, it is possible to evaluate solutions of differing enzyme activity. Using 10 cc. of the diluted potato enzyme solution described above, the effect of different quantities of substrate, and H_2O_2, and of various reaction times is shown in Figs. 2, 3, and 4. Figure 2 indicates that the use of larger quantities of substrate seems to give fairly constant responses within the limits tested. Forty milligrams was found satisfactory for the assay of this enzyme. Figure 3 indicates that larger quantities of H_2O_2 inhibit the activity of the peroxidase. Inhibition

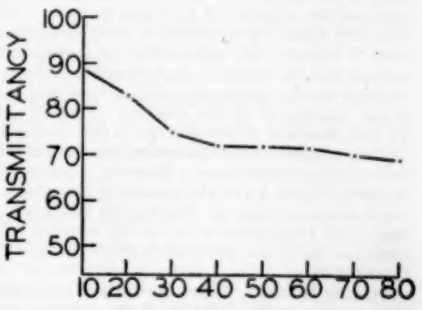

Fig. 2.—Effect of varied quantities of pyrogallol substrate on a solution assayed for peroxidase content.

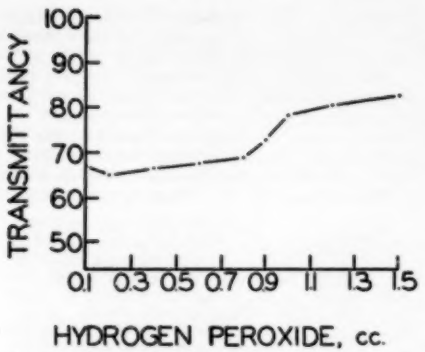

Fig. 3.—Effect of varied quantites of H_2O_2 on a solution assayed for peroxidase content.

was not considered to be of a significant degree when 0.8 cc. of 3% H_2O_2 was used. Figure 4 indicates that the effect of time is least critical beyond ten minutes. Small errors in time are less evident in the vicinity of ten minutes.

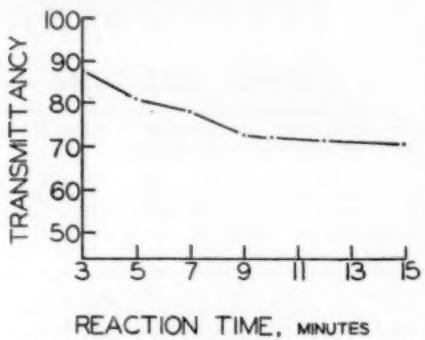

Fig. 4.—Effect of varied reaction times on a solution assayed for peroxidase content.

The same procedure was applied to potato enzyme B to quantitate the polyphenolase activity, omitting the addition of hydrogen peroxide. The area over which the logarithm of the transmittance bore a straight line relationship to quantity of enzyme was, in this case, quite limited. Above a certain quantity of enzyme solution there was consistent leveling-off of the reaction. Therefore, in all polyphenolase determinations it was necessary to keep all quantitative determinations within this area of linear comparison. However, using potato enzyme solution A and the peroxidase procedure, it was possible to maintain this straight line relationship at all concentrations within the areas of optimum reading of the spectrophotometer (above 10% transmittancy).

Enzyme Isolation.—These methods of assay were then applied to the isolation of the enzymic components of *Impatiens pallida* Nutt. and *Impatiens capensis* Meerb. Over 350 lb. of these species, more commonly known as jewelweed, were gathered in September of 1947 and 1948 from wild-growing plants of the Morton Arboretum and the University of Illinois Drug Plant Experiment Station at Lisle, Ill. The fresh material was found to be most satisfactorily preserved by expressing the juice in a hydraulic press immediately after gathering, and freezing the expressed juice in sealed, air-tight, plastic bags stored in a frozen food locker. The enzymes were preserved almost entirely over a period of a year. Tests were not made beyond this time.

Qualitative tests proved that the juice of jewelweed contained peroxidase and polyphenolase components. Tyrosinase was not present. Most of the basic work in characterizing the properties of the enzyme components was concerned with the polyphenolase enzyme. The attributed activity of the juice, due to enzymes, in the treatment of Rhus dermatitis would be due to the polyphenolase in the absence of hydrogen peroxide naturally occurring in the juice.

With the use of the spectrophotometric processes described above, it was readily possible to determine certain critical characteristics of the polyphenolase enzyme and the applicability of some of the more common steps of enzyme isolation to its purification. The raw juice was diluted 1:3 with distilled water for all tests. The optimum *p*H for the enzyme action was 7.3. It was completely inactivated by boiling for three minutes. It could best be salted out at 0.6 saturation with $(NH_4)_2SO_4$. It was precipitated with an equal quantity of acetone. There was little difference in polyphenolase activity between the two species. Enzyme activity is retained by preserving the juice with $CHCl_3$ or sodium benzoate, by lyophilization, or by freezing the raw juice. It is not adsorbed on kaolin, and a large proportion is lost in adsorption on siliceous earth and on bentonite. Aluminum hydroxide is the most efficient adsorptive agent if the enzyme is adsorbed at *p*H 5.3 and eluted at *p*H 8.0.

The determination of the basic properties of the polyphenol oxidase of the species of *Impatiens* as described made possible the integration of these characteristics into a method of isolation. All the studies previously accomplished dealt with the impure enzyme contained in the plant juice. The possible effects of steps used in isolating the enzyme were re-evaluated using partially purified extracts. The impurities removed by these processes were correlated with changes in enzyme activity and in the content of solids in the solutions studied.

Investigations described sought a method for producing a solution of *Impatiens* enzyme of sufficient purity and concentration for an evaluation of the action of the enzyme upon the irritant principle of poison ivy and an appraisal of the therapeutic efficacy of this action in experimental poison ivy dermatitis.

The isolation processes briefly outlined below followed the procedures mentioned above as being most applicable, using the raw juice diluted 1:3 with distilled water. Aluminum hydroxide adsorption was carried out at *p*H 5.3 using *p*H of 8.0 for elution with the aid of $Na_2HPO_4 \cdot 7H_2O$. Quantities of enzyme solution were mixed with equal volumes of $Al(OH)_3$ suspension. Products of elution were dialyzed free of salts in the refrigerator. The salting-out process with $(NH_4)_2SO_4$ utilized 0.6 saturated mixtures. The resulting precipitate was dis-

solved in distilled water and dialyzed free of salts. Precipitation with acetone was accomplished using equal amounts of enzyme solution and acetone. A better solution of the precipitate was obtained by making the mixture slightly alkaline.

A curve of areas indicating linear response of activity to concentration of each initial sample of juice was first determined and the products resulting from the various isolation steps were brought to known volumes. The amount of solution used in these assays was representative of a definite amount of original solution so that they could be referred to it directly in terms of corresponding activity of cubic centimeters of original solution. All assays were made in duplicate or until repeated values within 2% transmittancy of each other were obtained.

Dry weight determinations were made on representative samples of solutions of definite volume dried to constant weight at 110°. Results were calculated by the dilution factor to be directly comparable to the original weight of a definite volume of the initial jewelweed solution in order to evaluate the efficiency of the process or series of processes employed.

Process A involved precipitation of the enzyme with acetone, adsorption on, and elution from, $Al(OH)_3$, and salting-out with $(NH_4)_2SO_4$. This series of steps removed 99.7% of solids, but 98.7% of polyphenolase and 95.3% of total enzyme activity was also removed. Total enzyme activity was considered that amount of activity measured by the peroxidase quantitative test since the polyphenolase was also present, contributing to the oxidation. The advisability of introducing the latter two steps at such a loss of enzyme activity was questionable in view of the fact that in the initial precipitation 98.9% of solids was removed and there was a loss of 80.0% of polyphenolase and only 56.6% loss of total enzyme activity.

Process B consisted of adsorption on and elution from $Al(OH)_3$, salting-out with $(NH_4)_2SO_4$, then precipitation with acetone. The salting-out followed by adsorption on $Al(OH)_3$ was responsible for a complete loss of enzyme activity.

Process C followed the sequence of adsorption on and elution from $Al(OH)_3$ followed by dialysis to eliminate salts. The resulting solution was re-adsorbed on, and eluted from, $Al(OH)_3$. There was a loss of 99.2% of solids and a loss of 91.0% of polyphenolase, and a 90.0% loss of total enzyme activity. The great loss of enzyme activity occurred in the initial process.

The effect of repeated salting-out (process D) was next investigated. A portion of the jewelweed solution was salted out with $(NH_4)_2SO_4$, redissolved and dialyzed. The slightly turbid liquid so obtained was filtered through purified siliceous earth. On testing the solution before and after filtration, it was found that very little enzyme activity was lost by filtration. The solution was again salted out and dialyzed. The supernatant liquid from the salting-out process was brought to 0.8 saturation and the small precipitate was collected by high-speed centrifugation. In these processes it was possible to recover 32.7% of total enzyme activity, 31.3% of polyphenolase activity, and to eliminate 98.3% of solids.

Based on the information obtained by these preliminary processes, a large scale isolation and concentration was accomplished. The raw juice was diluted 1:3 with distilled water, centrifuged, and the supernatant treated as follows: salted-out by bringing to 0.6 saturation with $(NH_4)_2SO_4$, redissolved, filtered through siliceous earth, again salted-out with $(NH_4)_2SO_4$, dialyzed, adsorbed, and eluted from $Al(OH)_3$. The solution was one-sixtieth its original volume. This solution was assayed for total enzyme activity (peroxidase assay) and the area of linearity of activity curve determined. Assays using a solution prepared by diluting one part of solution with three parts of water were accomplished. One cubic centimeter of this solution was assayed for total oxidative activity in the presence of H_2O_2 following the process for total enzyme activity determination. Using water as the comparator, this product gave a transmittancy of 14.5%. A transmittancy of 37.8% was obtained for a similarly treated sample of the diluted raw jewelweed juice. This indicated that the final enzyme product contained a greater total oxidizing power toward pyrogallol than raw jewelweed juice. This final enzyme product was diluted until its total oxidative power approximated that of the raw jewelweed juice, giving a transmittancy of 39.5% when similarly diluted for assay. This was assayed for polyphenolase activity (56.6%) and total enzyme activity (44.7%) using 1-cc. quantities. Equivalent quantities of the jewelweed solution gave a polyphenolase activity of 87.0% transmittancy and total enzyme activity of 60.1% transmittancy. The small differences in transmittancies of assays of the enzyme solution for total oxidation color (39.5%) and total enzyme activity (44.7%) as compared to the large difference in the case of the raw juice (37.8 and 60.1%) illustrated the loss of nonenzymic oxidants. The purified enzyme solution was almost wholly enzymic, as illustrated by this small difference between total oxidative and total enzymic oxidation transmittancies.

The dry weight of the final enzyme solution represents only 0.01% of the solids present in raw jewelweed juice. This enzyme solution was considered to represent sufficient purification and isolation of the enzymes for the study of their activity in the treatment of experimental Rhus dermatitis (17).

SUMMARY AND CONCLUSIONS

Accurate and rapid methods for the quantitative analysis of peroxidases and polyphenolases have been found which allow for mechanical deduction of nonenzymic oxidation effects commonly found in crude plant extracts. These methods make use of the Beckman Spectrophotometer, a more accurate instrument than formerly used in assays of this type. Use was made of these methods in evaluating the successive steps for a method of partial isolation of the enzymes present in two species of *Impatiens*. Partial isolation was accomplished using successive steps of salting-out and adsorption on, and elution from $Al(OH)_3$. An enzyme preparation was obtained which removed 99.99 per cent, of solids and had an enzymic

oxidative power toward polyphenols approximately equivalent to the total oxidative activity of the raw juice. By this means of isolation it was possible to obtain an enzyme of sufficient purity for studies in the pharmacological activity of phenol-oxidizing enzymes and to determine the role they play in the reputed activity of the juice of species of *Impatiens* in the treatment of Rhus dermatitis.

A modification of previous methods of assay for peroxidases and polyphenolases is presented which provides for more accurate colorimetric quantitative determination. A method of isolating enzymes of this type is presented utilizing the new methods of assay whereby the accuracy of each step in the isolation is followed. This constitutes the initial work on the isolation of the enzymes of the species of *Impatiens*.

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The Synthesis of Certain Disubstituted Piperidines*

By R. O. BACHMANN† and GLENN L. JENKINS

Several isomers of 2,6-bis-(1-hydroxyalkyl) piperidine hydrochlorides, in which the alkyl group is propyl, butyl, pentyl, hexyl, and benzyl have been prepared by hydrogenating the corresponding five 2,6-diacylpyridine intermediates. 3,5-Diphenylpiperidine and a by-product, 2,4-diphenylpentamethylenediamine dihydrochloride, were also prepared. The disubstituted piperidines are to be tested for pharmacological activity.

CERTAIN 3,5-dialkylpiperidines, certain 2,6-bis-(1-hydroxyalkyl)piperidines and 2,6-bis-(phenylhydroxymethyl)piperidine possess in duplicate the structural arrangement common to compounds known to have sympathomimetic activity. The purpose of this work was to synthesize some of these symmetrically disubstituted piperidines so that they might be tested for possible pharmacological activity.

3,5-Diphenylpiperidine and 2,4-diphenylpentamethylenediamine were prepared by catalytic reduction of 2,4-diphenylglutaronitrile. Adkins (1) reported a similar reduction of trimethylene cyanide to piperidine and cadaverine.

Certain 2,6-bis-(1-hydroxyalkyl)piperidines, and 2,6-bis-(phenylhydroxymethyl)piperidine were prepared by catalytic reduction of 2,6-diacylpyridines. The 2,6-diacylpyridines were prepared by the reaction of a suitable alkyl cadmium chloride with 2,6-pyridinedicarboxylic acid chloride (see page 45).

Considerable difficulty was encountered in the catalytic reductions of the hydrochlorides of 2,6-dipropionylpyridine and 2,6-dipentanoylpyridine. On the other hand, the hydrochlorides of 2,6-dibutanoylpyridine, 2,6-dihexanoylpyridine and 2,6-dibenzoylpyridine were more readily reduced. Since the 2,6-disubstituted piperidines possess four asymmetric carbon atoms of the ABBA type (2), they should exist as four *dl* mixtures and two *meso* forms. In general each reduction yielded not more than three isomeric forms. This is in conformity with the work on catalytic hindrance

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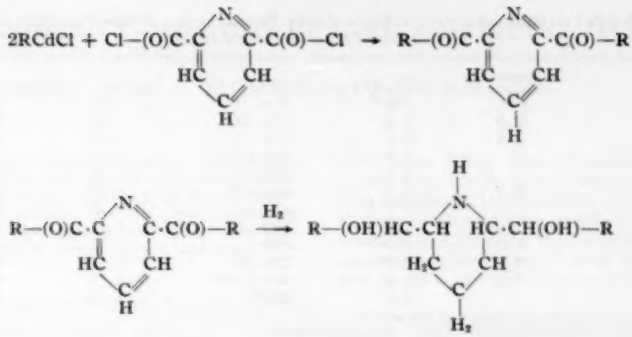

by Linstead, *et al.* (3). The nature of these isomers has not been determined, but further work is contemplated.

EXPERIMENTAL

3,5-Diphenylpiperidine and 2,4-Diphenylpentamethylenediamine.—Fourteen grams of 2,4-diphenylglutaronitrile (4, 5) in 100 cc. of dioxan was shaken with 0.7 Gm. of Raney nickel and hydrogen at 2,500 lb./sq.in. and 200° for five hours. The reaction mixture was filtered, stripped of dioxan, and distilled under reduced pressure: boiling range 200–238°/15 mm.; yield, 10 Gm. Redistillation: boiling range 169–179°/2 mm. The distillate was converted to the hydrochlorides. The hydrochlorides were fractionally crystallized from absolute isopropyl alcohol in a vacuum desiccator. The first crystalline fraction was recrystallized from absolute isopropyl alcohol yielding 3 Gm. of 2,4-diphenylpentamethylenediamine dihydrochloride, m.p. 319–320° (aluminum block). The second fraction was recrystallized from an isopropyl alcohol-ether mixture yielding 1 Gm. of 3,5-diphenylpiperidine hydrochloride, m.p. 231–232°.

Anal.—Calcd. for $C_{17}H_{24}N_2Cl_2$: N, 8.55%. Found: N, 8.42%. Calcd. for $C_{17}H_{20}NCl$: N, 5.11%. Found: N, 5.14%.

Synthesis of 2,6-Diacylpyridines.—The 2,6-diacylpyridines listed in Table I were prepared by a procedure similar to that of Cason and Prout (6) for the preparation of ketones from acid chlorides. In general, a previously prepared alkyl cadmium chloride was treated with 2,6-pyridinedicarboxylic acid chloride in anhydrous thiophene-free benzene. The acid chloride was prepared by the procedure of Wolffenstein and Hartwich (7) from 2,6-pyridinedicarboxylic acid (8) and thionyl chloride. Where the diacylpyridines were oils, they were redistilled until colorless. The 2,4-dinitrophenylhydrazones of the 2,6-diacylpyridines were prepared by the procedure of Shriner and Fuson (9).

2,6-Bis-(1-hydroxyalkyl)piperidines and 2,6-Bis-(phenylhydroxymethyl)piperidines.—The substituted piperidine hydrochlorides listed in Table II were prepared by dissolving 0.015 to 0.02 mole of a diacylpyridine in 50 to 100 cc. of absolute ethanol, adding an equivalent amount of dry hydrogen chloride and 0.1 to 0.2 Gm. of platinum oxide catalyst, and shaking for twenty-four hours with hydrogen at room temperature and 2 to 3 atmospheres pressure in a Parr hydrogenation apparatus (10). This procedure was described by Craig and Hixon (11). The hydrogenations did not proceed unless the ethanol solutions of the 2,6-diacylpyridines were previously shaken with Raney nickel. The hydrogenations did not proceed to completion even on prolonged treatment with hydrogen and repeated regeneration of the catalyst with oxygen (12). Attempts to hydrogenate in concentrated hydrochloric acid (13) or in the presence of ferrous chloride promotor failed. In one instance 2,6-dipentanoylpyridine was successfully hydrogenated using Raney nickel and hydrogen at 100° and 100 atmospheres pressure for one hour.

The reaction mixtures were filtered and stripped of the ethanol. The remaining residues were fraction-

TABLE I.—DIACYLPYRIDINES PREPARED

Acyl-	B. r., °C./mm.	Yield, %	M.p., °C.	2,4-Dinitrophenylhydrazones[a] Nitrogen,[b] % Calcd.	Found
Propionyl-	120–125/1	22	272–273	22.86	22.82; 22.95
Butanoyl-	143–146/5	40	254–255	21.75	21.09; 21.25
Pentanoyl-	163–170/2	39	234–235	20.75	20.75; 20.91
Hexanoyl-	190–204/5	33	194–195	19.83	18.78; ...
Benzoyl-	180[d]	43	236–237	...	... ...

[a] All orange plates.
[b] Uncorrected. In all aluminum block.
[c] Analysis by Huffman Microanalytical Labs., Denver, Colo.
[d] M.p. from isopropyl alcohol. Wolffenstein and Hartwich (7) prepared 2,6-dibenzoylpyridine from benzene and 2,6-pyridinedicarboxylic acid chloride in the presence of aluminum chloride.

TABLE II.—2,6-BIS(1-HYDROXYALKYL)PIPERIDINE SALTS PREPARED BY HYDROGENATION OF 2,6-DIACYLPIPERIDINES USING PLATINUM OXIDE

Alkyl-	Starting Material, Gm.	Product,[a] Gm.	M.p., °C.[b]	Nitrogen[c], % Calcd.	Found
Propyl-	6.0	0.4	192–193	5.93	5.77; 5.72
Butyl-	5.5	2.5	173–175	5.27	5.35; 5.31
		1.0	165.5–167	5.27	5.39; 5.37
		0.3	148–149.5	5.27	5.02; 5.04
Pentyl-	3.5	0.3	203–205	4.77	5.05; 5.01
		0.1	130–131	4.77	4.55; 4.63
Pentyl-	3.5	1.1	119–120	4.77	4.54; 4.46
Pentyl-[g]	4.0	1.0[d]	292[e]	4.57	4.39; 4.54
Hexyl-	8.0	2.5[d]	286–287[e]	4.19	4.09; 4.23
Benzyl-[f]	5.0	2.4	288–290[d,e]	4.19	4.23; 4.24
		1.0	269[d,e]	4.19	4.18; 4.19

[a] As hydrochlorides unless indicated otherwise. All colorless needles.
[b] Uncorrected.
[c] Analysis by Huffman Microanalytical Labs., Denver, Colo.
[d] Sulfate.
[e] In aluminum block.
[f] 2,6-Bis-(phenylhydroxymethyl)piperidine hydrochloride.
[g] Reduced with Raney nickel.

ally crystallized from ethanol-ether mixtures. When the hydrochlorides were oils, the sulfate salts were made in absolute ether and recrystallized from ethanol.

SUMMARY

1. 3,5-Diphenylpiperidine hydrochloride, two isomeric 2,6-bis-(phenylhydroxymethyl)piperidine hydrochlorides and nine 2,6-bis-(1-hydroxyalkyl)piperidine hydrochlorides were prepared.

2. Four new 2,6-diacylpyridine intermediates were prepared, and 2,6-dibenzoylpyridine was prepared by a new method.

3. 2,4-Diphenylpentamethylenediamine dihydrochloride was obtained as a by-product.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Anthracene-2-sulfonic acid
2,3-Dihydroxyanthracene
Carbon tetraiodide
2,6-Epoxyhexane
3-Fluorophenylalanine
Camphane aldehyde
p-Methoxythiophenol
Maleimide
Ketomalic acid
3-Hydroxynorcholinic acid
Ionene
Lysergic acid
Neurine
Promulein
Protoporphyrin 111
Trypoflavin
Alloxazineadenine dinucleotide
D-Mannosamine
Capsaicin
Avidin

Chemical Constitution and Analgetic Action*

By M. B. SLOMKA† and F. W. SCHUELER

The relation between chemical constitution and analgetic action has been investigated in several compound types which bear a structural relationship to the morphine-type analgetics. Analgetic potency has been demonstrated in several compound types.

THE CONSIDERATION of the relationship between pharmacologic action and chemical constitution has, in recent years, led to an increased understanding of pharmacologic mechanisms and to the development of new pharmacologic agents (1–10).

The attempts to synthesize potent analgetic compounds without the addictive liabilities and other undesirable side effects of morphine are numerous, and, in general, have consisted of the screening of large numbers of compounds possessing one or more of the structural characteristics of the morphine molecule (11, 12). Methadone, meperidine, and Dromoran (Nu-2206) are notable examples of analgetic compounds that have resulted.

Schueler, Gross, and Holland (9) noted the "presence of both sympathomimetic and parasympathomimetic-like moieties connected (in general) by the same amino nitrogen atom" in the structures of morphine and its analogs, and further suggested that the analgetic action of the morphine type agents may be traceable to the presence of such moieties in the same molecule which may act in part through a peripheral analgetic mechanism.

On the basis of the above hypothesis, several types of compounds containing the aforementioned autonomic-like moieties were prepared, and their analgetic potencies determined.

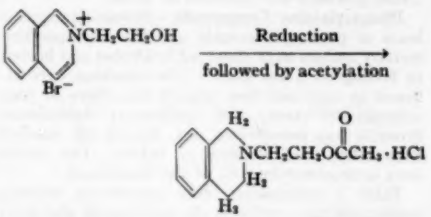
EXPERIMENTAL

Synthetic Procedures

β-Phenylethylamine Compounds.—Equimolecular amounts of the corresponding secondary amine and ω-bromoalkyl ester were mixed. If the reaction did not proceed spontaneously, the mixture was warmed on the steam bath for one to two hours. An equal volume of absolute ether was then added and the crystalline precipitate of the secondary amine hydrobromide removed by filtration and washed with more ether. Dry hydrogen chloride was passed into the combined filtrate and washings. The precipitated hydrochlorides of the alkoxyethyl-β-phenylethylamines were collected by filtration in yields of 20–40% of the theoretical and purified by repeated solution in alcohol and reprecipitation with ether until a constant melting point was attained.

Isoquinoline and Tetrahydroisoquinoline Compounds.—The isoquinolinium compounds were prepared by warming equimolecular amounts of redistilled isoquinoline and the corresponding bromoalcohol or ester on a hot plate for one to two hours. After allowing the mixture to stand overnight, an equal volume of absolute ether was added, the crystals filtered off and washed with more ether. The yields were approximately 70% and the products were purified as before.

N-Acetoxyethyl tetrahydroisoquinoline hydrochloride was prepared according to the following scheme:



Following the procedure of Feer and Koennigs (13), 40 Gm. of granular tin was mixed with 19 Gm. of N-(2-hydroxyethyl) isoquinolinium bromide, and 120 cc. of concentrated hydrochloric acid was added. The mixture was heated on the steam bath, and an additional 120 cc. of acid was added in small portions over a two-hour period, during which time the tin dissolved. The solution was diluted eightfold with water and made distinctly alkaline to litmus with 20% sodium hydroxide solution. The precipitate of tin hydroxide was filtered off, washed twice with water, and extracted twice with 200-cc. portions of ether. The other extracts were filtered and the ether evaporated off on the steam bath, leaving behind a brownish-yellow oil. This was dissolved in 10 volumes of dry ether, the solution cooled, and excess acetyl chloride added. A bright purple crystalline precipitate formed which was filtered off and washed with ether. The product was purified as before until a colorless product of constant melting point was obtained. The yield was 50% of the calculated yield.

Dialkylaminomethylcyclohexane Compounds.—These compounds were prepared from the corresponding 2-dialkylaminomethyl-cyclohexan-1-one compounds in accordance with the following scheme:

* Received July 6, 1950, from the Department of Pharmacology, College of Medicine, State University of Iowa.
Abstract presented at the Atlantic City meetings of the Federation of American Societies for Experimental Biology, April, 1950.
† Fellow of the American Foundation for Pharmaceutical Education.

TABLE II.—2,6-BIS(1-HYDROXYALKYL)PIPERIDINE SALTS PREPARED BY HYDROGENATION OF 2,6-DIACYLPIPERIDINES USING PLATINUM OXIDE

Alkyl-	Starting Material, Gm.	Product,[a] Gm.	M.p., °C.[b]	Nitrogen[c], % Calcd.	Found
Propyl-	6.0	0.4	192–193	5.93	5.77; 5.72
Butyl-	5.5	2.5	173–175	5.27	5.35; 5.31
		1.0	165.5–167	5.27	5.39; 5.37
		0.3	148–149.5	5.27	5.02; 5.04
Pentyl-	3.5	0.3	203–205	4.77	5.05; 5.01
		0.1	130–131	4.77	4.55; 4.63
Pentyl-	3.5	1.1	119–120	4.77	4.54; 4.46
Pentyl-[g]	4.0	1.0[d]	292[e]	4.57	4.39; 4.54
Hexyl-	8.0	2.5[d]	286–287[e]	4.19	4.09; 4.23
Benzyl-[f]	5.0	2.4	288–290[d,e]	4.19	4.23; 4.24
		1.0	269[d,e]	4.19	4.18; 4.19

[a] As hydrochlorides unless indicated otherwise. All colorless needles.
[b] Uncorrected.
[c] Analysis by Huffman Microanalytical Labs., Denver, Colo.
[d] Sulfate.
[e] In aluminum block.
[f] 2,6-Bis-(phenylhydroxymethyl)piperidine hydrochloride.
[g] Reduced with Raney nickel.

ally crystallized from ethanol-ether mixtures. When the hydrochlorides were oils, the sulfate salts were made in absolute ether and recrystallized from ethanol.

SUMMARY

1. 3,5-Diphenylpiperidine hydrochloride, two isomeric 2,6-bis-(phenylhydroxymethyl)piperidine hydrochlorides and nine 2,6-bis-(1-hydroxyalkyl)piperidine hydrochlorides were prepared.

2. Four new 2,6-diacylpyridine intermediates were prepared, and 2,6-dibenzoylpyridine was prepared by a new method.

3. 2,4-Diphenylpentamethylenediamine dihydrochloride was obtained as a by-product.

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(10) "Organic Syntheses," John Wiley and Sons, New York, 1941, Coll. vol. I, pp. 61–67.
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(12) Carothers, W. H., and Adams, R., *ibid.*, **45**, 1071 (1923).
(13) Crook, K. E., and McElvain, S. M., *ibid.*, **52**, 4006 (1930).

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Anthracene-2-sulfonic acid
2,3-Dihydroxyanthracene
Carbon tetraiodide
2,6-Epoxyhexane
3-Fluorophenylalanine
Camphane aldehyde
p-Methoxythiophenol
Maleimide
Ketomalic acid
3-Hydroxynorcholinic acid
Ionene
Lysergic acid
Neurine
Promulein
Protoporphyrin 111
Trypoflavin
Alloxazineadenine dinucleotide
D-Mannosamine
Capsaicin
Avidin

Chemical Constitution and Analgetic Action*

By M. B. SLOMKA† and F. W. SCHUELER

The relation between chemical constitution and analgetic action has been investigated in several compound types which bear a structural relationship to the morphine-type analgetics. Analgetic potency has been demonstrated in several compound types.

THE CONSIDERATION of the relationship between pharmacologic action and chemical constitution has, in recent years, led to an increased understanding of pharmacologic mechanisms and to the development of new pharmacologic agents (1–10).

The attempts to synthesize potent analgetic compounds without the addictive liabilities and other undesirable side effects of morphine are numerous, and, in general, have consisted of the screening of large numbers of compounds possessing one or more of the structural characteristics of the morphine molecule (11, 12). Methadone, meperidine, and Dromoran (Nu-2206) are notable examples of analgetic compounds that have resulted.

Schueler, Gross, and Holland (9) noted the "presence of both sympathomimetic and parasympathomimetic-like moieties connected (in general) by the same amino nitrogen atom" in the structures of morphine and its analogs, and further suggested that the analgetic action of the morphine type agents may be traceable to the presence of such moieties in the same molecule which may act in part through a peripheral analgetic mechanism.

On the basis of the above hypothesis, several types of compounds containing the aforementioned autonomic-like moieties were prepared, and their analgetic potencies determined.

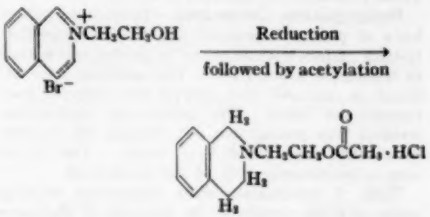
EXPERIMENTAL

Synthetic Procedures

β-Phenylethylamine Compounds.—Equimolecular amounts of the corresponding secondary amine and ω-bromoalkyl ester were mixed. If the reaction did not proceed spontaneously, the mixture was warmed on the steam bath for one to two hours. An equal volume of absolute ether was then added and the crystalline precipitate of the secondary amine hydrobromide removed by filtration and washed with more ether. Dry hydrogen chloride was passed into the combined filtrate and washings. The precipitated hydrochlorides of the alkoxyethyl-β-phenylethylamines were collected by filtration in yields of 20–40% of the theoretical and purified by repeated solution in alcohol and reprecipitation with ether until a constant melting point was attained.

Isoquinoline and Tetrahydroisoquinoline Compounds.—The isoquinolinium compounds were prepared by warming equimolecular amounts of redistilled isoquinoline and the corresponding bromoalcohol or ester on a hot plate for one to two hours. After allowing the mixture to stand overnight, an equal volume of absolute ether was added, the crystals filtered off and washed with more ether. The yields were approximately 70% and the products were purified as before.

N-Acetoxyethyl tetrahydroisoquinoline hydrochloride was prepared according to the following scheme:



Following the procedure of Feer and Koennigs (13), 40 Gm. of granular tin was mixed with 19 Gm. of N-(2-hydroxyethyl) isoquinolinium bromide, and 120 cc. of concentrated hydrochloric acid was added. The mixture was heated on the steam bath, and an additional 120 cc. of acid was added in small portions over a two-hour period, during which time the tin dissolved. The solution was diluted eightfold with water and made distinctly alkaline to litmus with 20% sodium hydroxide solution. The precipitate of tin hydroxide was filtered off, washed twice with water, and extracted twice with 200-cc. portions of ether. The other extracts were filtered and the ether evaporated off on the steam bath, leaving behind a brownish-yellow oil. This was dissolved in 10 volumes of dry ether, the solution cooled, and excess acetyl chloride added. A bright purple crystalline precipitate formed which was filtered off and washed with ether. The product was purified as before until a colorless product of constant melting point was obtained. The yield was 50% of the calculated yield.

Dialkylaminomethylcyclohexane Compounds.—These compounds were prepared from the corresponding 2-dialkylaminomethyl-cyclohexan-1-one compounds in accordance with the following scheme:

* Received July 6, 1950, from the Department of Pharmacology, College of Medicine, State University of Iowa.
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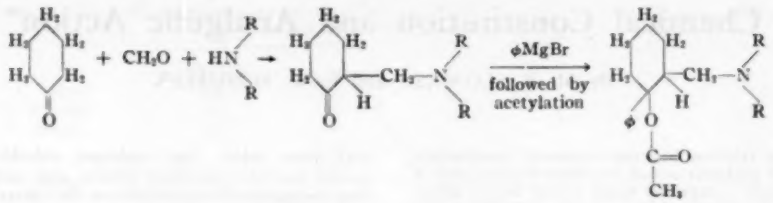


The 2-dialkylaminomethyl-cyclohexan-1-one compounds were prepared in the manner previously reported by Mannich (14, 15).

The 2-dialkylaminomethyl-cyclohexan-1-one compound (0.12 mole) in absolute ether was added with constant stirring to an ether solution of phenyl magnesium bromide prepared from 0.5 mole of magnesium and 0.2 mole of bromobenzene. On completion of the addition, the mixture was refluxed for one-half hour, poured over ice, and made alkaline to litmus with cold 20% sodium hydroxide solution. The 2-dialkylaminomethyl-cyclohexan-1-one was extracted with ether, the ether solution dried over sodium hydroxide sticks, filtered, and an excess of acetyl chloride added. A white precipitate of the 1-phenyl-1-acetoxy-2-dialkylaminomethyl-cyclohexane hydrochloride separated out in 30% yield. These products were purified as before.

Phenacylamino Compounds.—Molecular equivalents of phenacyl bromide and the corresponding tertiary amines were dissolved in alcohol and heated to boiling on a hot plate. The solutions were allowed to cool and then poured into three or four volumes of ether. The quaternary ammonium bromide was precipitated out, filtered off, washed with ether, and purified as before. The yields were approximately 50% of the theoretical.

Table I summarizes data concerning melting points and the results of the analyses of the compounds synthesized.

Analgetic Tests and Toxicity Determinations

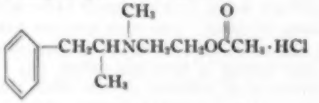
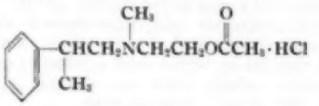
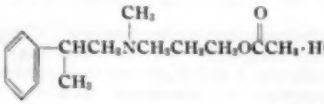
Analgetic potencies were determined on Sprague-Dawley strain albino rats, weighing from 80–150 Gm., twenty minutes after intraperitoneal administration of the compound in physiological saline solution, using a modification of the D'Amour and Smith technique (16). Groups of 10 to 20 animals were used on each dosage level. The Null hypothesis and Student's T were used to determine the significance of the results. Toxicities were determined on albino mice and the LD_{50}'s were calculated using the method of probit analysis. Twenty to forty animals were used on each dosage level. Table II summarizes the data from the analgetic and toxicity tests.

DISCUSSION

The yield of active compounds on the basis of the hypothesis in this preliminary study is seen to be greater than 50%, thereby suggesting the usefulness of the hypothesis as a means of approaching the synthesizing of materials in this pharmacological class. The hypothesis, however, is not sufficiently refined to explain the inactivity of the β-phenyl-β-methyl derivative (A2), synthesized from Vonedrine, as compared with the activity of the β-phenyl-α-methyl derivative (A1), synthesized from desoxyephedrine, or the activity conferred by the inclusion of an additional carbon in the parasympathomimetic moiety of compound A3.

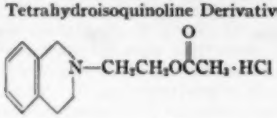
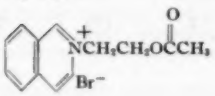
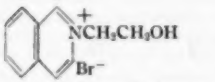
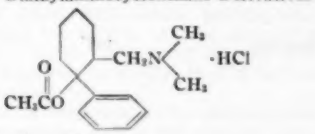
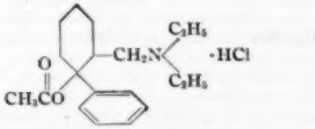
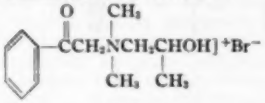
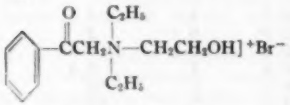
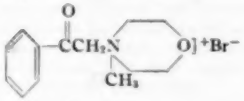
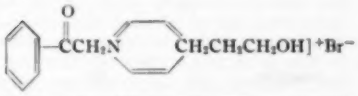
The effect of cyclization of the alkylamino chain of the sympathomimetic moiety is seen in the tetrahydroisoquinoline compound (B1). The increased activity of this compound over that of the phenylethylamine series may indicate the desirability of retaining the intact piperidine ring. Rupture of the piperidine ring in morphine leads to a virtually complete loss of analgetic action (17);

TABLE I.—PHYSICAL CONSTANTS

Compound	Element Calculated, %	Element Analyzed, %	Melting Point, °C.
(A) Phenylethylamine Derivatives			
(1) 	12.36 Cl	13.10 Cl[a]	106–107
(2) 	12.36 Cl	13.25 Cl	133–134
(3) 	11.80 Cl	12.29 Cl	228–230

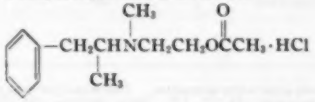
(*Continued on p. 49*)

TABLE I.—PHYSICAL CONSTANTS (*Continued*)

(B)	Tetrahydroisoquinoline Derivatives			
(1)		61.02 C 7.01 H	59.60 C[b] 7.03 H[b]	181–182
(2)		26.93 Br	27.10 Br	121–122
(3)		52.00 C 4.77 H	52.09 C 4.69 H	156
(C)	Dialkylaminocyclohexane Derivatives			
(1)		65.55 C 8.32 H	64.10 C 8.15 H	202–203
(2)		67.13 C 8.81 H	68.29 C 9.30 H	181.5–182.5
(D)	Phenacylamine Derivatives			
(1)		26.44 Br	26.10 Br	210–211
(2)		25.29 Br	25.50 Br	210–211
(3)		26.62 Br	26.10 Br	203
(4)		23.77 Br	23.35 Br	179–181

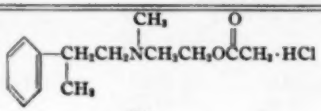
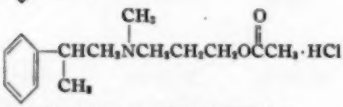
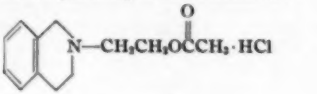
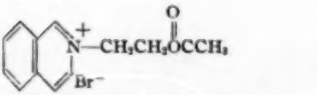
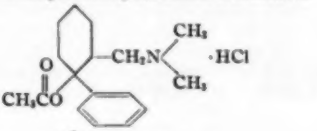
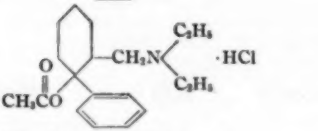
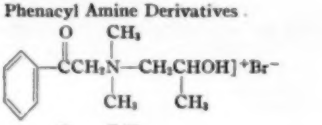
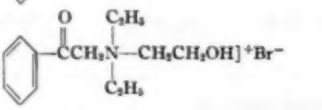
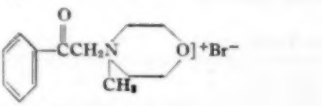
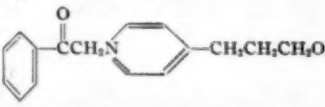
[a] Halogen analysis by the Volhard method.
[b] Carbon and hydrogen analysis by the Clark Microanalytical Laboratory, Urbana, Ill.

TABLE II.—ANALGETIC TESTS

Compound	Mean % Increase in Time for Tail Twitch	Confidence Limit, %	Dosage, Mg./Kg.	LD_{50} (mice), Mg./Kg.
(A) Phenylethylamine Derivatives				
(1) 	34.3	1	100	149

(*Continued on p. 50*)

TABLE II.—ANALGETIC TESTS (*Continued*)

(2)		Inactive	...	200	247
(3)		17.2	1	100	269
(B)	Tetrahydroisoquinoline Derivatives				
(1)		44.6	2	75	302
(2)		Inactive	...	100	...
(C)	Dialkylaminocyclohexane Derivatives				
(1)		Inactive	...	100	...
(2)		17.6 52.5	1 1	25 50	68 ...
(D)	Morphine Derivatives				
(1)	Morphine Sulfate	102.7	1	2	...
(2)	Morphine Methiodide[a]	103.0[b]	0.1	2	...
(3)	Heroine Sulfate	32.3	0.1	0.5	...
(4)	Heroine Methiodide[a]	16.5	1	0.5	...
(5)	Codeine Sulfate	89.3	1	10	...
(6)	Codeine Methiodide[a]	14.4	2	10	...
(E)	Phenacyl Amine Derivatives				
(1)		Inactive	...	25	...
(2)		Inactive	...	10	..
(3)		Inactive	...	50	..
(4)		Inactive	...	50	...

[a] Kindly supplied by Dr. Lyndon F. Small.

[b] Maximum response observed at twenty-, forty-, or sixty-minute periods after administration. The mean per cent increases at each of these time periods and their confidence limits are: twenty minutes—53.7%, 1%; forty minutes—84.2%, 0.1%; sixty minutes—61.2%, 1%.

this is also true for the Demerol analogs (18). The isoquinolinium analog is seen to be inactive.

In the structures of the dialkylaminomethylcyclohexane compounds, it should be noted that both moieties share the same alkyl chain and that a presumably inert cyclical nucleus has been added to the molecule. It was surprising to find the dimethylamino compound (C1) inactive in view of the activity of the diethylamino analog (C2), especially in regard to the greater physiological activity of methyl analogs in most homologous series of analgetic agents.

All these compounds appear to be central stimulants producing clonic convulsions, which precluded further testing in dosages higher than those reported in Table II. Their toxicity for the rat appears to be less than that for the mouse. In preliminary experiments, in an attempt to control the central stimulant effects with sodium pentobarbital, it appears that the toxicity of these compounds is increased. Possibly stimulation on one level is masking depression on another level of the central nervous system.

In order to determine if the quaternization of the amino nitrogen atom or the aromatic character of the isoquinoline ring was responsible for the inactivity of compound B2 as compared with B1, the analgetic potencies of morphine, heroine, and codeine and their quaternary methyl iodides were determined. The effect of quaternization varied in each case: morphine showing no decrease in potency, heroine a marked decrease, and codeine a still greater decrease at the dosage levels tested. Eddy has found a striking diminution in the analgetic effect of morphine methochloride in cats (19) and has found that morphine methiodide has no significant analgetic effect in mice at dosages of 5–100 mg./Kg. (20). It appears that although quaternization decreases analgetic activity, it does not destroy it.

The quaternary phenacyl compounds (E1–E4) containing the sympathomimetic moiety similar to that found in Kephrine were found to be inactive in the dosage administered and the clonic convulsions resulting from higher dosage prevented further testing. From preliminary experiments on dogs, compounds of this last type appear to possess respiratory stimulant properties.

The use of the terms sympathomimetic and parasympathomimetic moieties in any fuller sense than to point out spatial or structural similarity must be done with the greatest caution in view of the following considerations. The necessary and sufficient chemical constitutional requirements for sympathomimetic and parasympathomimetic action have not been established. Indeed, the term sympathomimetic (and parasympathomimetic) implies a large number of different type actions, the qualitative and quantitative nature of which depends on the biological test object on which the action is studied. Furthermore, little is known as yet as to the mechanism of the analgetic response, particularly as to whether it results from drug action at a unique and distinct site or whether it is a resultant effect of action at several different sites. Finally, and of paramount importance in the consideration of chemical constitution and pharmacologic action, there is as yet no justification for the assumption that the combination of so-called sympathomimetic and parasympathomimetic moieties, combined in the manner of this study, results in the retention of any autonomic character by the molecule. The pharmacodynamic characteristics of the molecules may represent emergent actions reflecting the chemical joining of the given autonomic-like moieties, and may give rise to a new moiety. We possess, as yet, little information concerning the mode of emergence of new moieties through combination of given moieties.

It is believed that the essential advantage in this type of consideration of the chemical constitutional requirements of analgetic agents is in the direction it may give toward further synthesis of compounds of interest in this series, and not as substantiating evidence that the autonomic nervous system is necessarily involved in the analgetic mechanism.

SUMMARY

1. Twelve new compounds were prepared and their analgetic potencies determined on rats. The LD_{50}'s of five of these compounds were determined on mice.

2. The analgetic potencies of morphine, heroine, and codeine were compared with those of their quaternary methyl analogs on rats.

3. The results of this investigation illustrate the usefulness of working hypotheses based upon the consideration of the relationship between chemical constitution and physiologic action.

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The Laxative Activity in Mice of the Various Parts of the Senna Plant*

By I. W. GROTE and MARIBELLE WOODS

Using a standardized procedure previously reported for determining the laxative activity in mice of senna, the laxative potency of various grades and kinds of commercial Tinnevelly and Alexandria senna leaves and Tinnevelly senna pods have been found to be reasonably uniform, the variations in potency not exceeding 25 per cent of the mean. Tinnevelly senna was successfully grown to maturity in Chattanooga, Tenn., as well as on a larger scale in the Imperial Valley of California. The leaves and pods of such experimentally grown senna plants were found to have approximately the same potency as those from India or Egypt. The petioles were found to possess about 75 per cent of this potency while the stems and roots had only 50 per cent.

TINNEVELLY SENNA, which is produced from extensive cultivation of *Cassia angustifolia* Vahl in southern India, has been successfully grown to maturity in Chattanooga, Tenn., Valdosta, Ga., and in the Imperial Valley of California. These experimental growths of a botanical drug which is used in such great annual tonnage were prompted by the rapidly diminishing supply in the warehouses in this country during World War II.

Some of the difficulties of growing Tinnevelly senna in this country are worth reporting at this time. The first trial planting of seed from commercially obtained pods failed to germinate. A small supply of viable seed was finally obtained from freshly imported pods by Dr. E. N. Gathercoal, Emeritus Professor of Pharmacognosy, University of Illinois. Later a quantity adequate for several experimental plantings was secured by salvaging seed from the waste obtained in commercial cleaning of senna leaflets. However, the germination of these seeds was poor. Experimental plots of the drug were planted in Chattanooga, and at Valdosta, Ga., in 1943 and again in 1947, under the direction of Mr. Elsworth Brown of our staff. Larger plots were planted under the direction of Dr. Gathercoal in the Imperial Valley of California in 1943 and following years.

EXPERIMENTAL

The plant matured about ninety days after planting, but was found in Chattanooga to be completely "winter killed" when exposed to a temperature slightly below 50° F. for even one night (1). A temperature of 45–48° F. caused the leaves to turn brown and drop off, and the entire plant to die quickly thereafter. Since the active principles of the senna leaves are soluble in water, it is desirable that there be little rainfall during the season of approaching maturity of the plant. Examination of the weather records by Dr. Gathercoal revealed that the Imperial Valley was the most suitable place in the United States where these conditions could be expected. A seed supply sufficient to plant six acres was finally built up by harvesting seed from the experimentally grown material.

The California- and Tennessee-grown senna closely conformed to typical color characteristics, but that from Valdosta in southern Georgia was mildewed and otherwise discolored by rain at harvest time, and hence disqualified for our studies.

Rail freight rates from California to the eastern part of the United States exceed transportation by boat from India or Egypt. This expense added to higher labor makes the cost of delivered senna a few cents more per pound than the normal peacetime price.

Since we had available the entire senna plant of domestic growth, it seemed interesting and feasible to evaluate the relative laxative effect of the leaflets, pods, petioles, stems, and roots, as well as to compare these with the commercially available material. According to our standardized method of assay (2), the finely ground drug was suspended in boiling distilled water and 0.5-cc. doses were administered to mice by stomach tube. The results were considered positive if the feces were softened sufficiently to stain the underside of newsprint paper, and negative if no stain was visible. Our reference standard was from a lot of senna which had been selected several years ago for good green color and representative laxative action. The results are shown in Table I.

Our work seems to confirm the literature reports (3) in regard to the relative activity of the leaflets and the pods, which may vary from 70 to 115% of the senna selected as our standard for activity. This same range of activity is covered by different samples of leaflets, consequently the pods may be equal in strength to some leaflets, stronger or weaker than some other leaflets. With the domestically grown Tinnevelly pods, we would conclude that the pods are slightly more active than the leaflets, the petioles about 75% as active, whereas the stems were 50% as active, and the roots 55% as active as the leaflets.

* Received May 5, 1950, from the Research Laboratories, Chattanooga Medicine Company, Chattanooga, Tenn.
Presented to the Scientific Section, A. Ph. A., Atlantic City meeting, May, 1950.

TABLE I.—LAXATIVE ACTIVITY IN MICE OF VARIOUS PARTS OF THE TINNEVELLY SENNA PLANT

	M.La.D.$_{50}$ Sample,[a,b] Mg./Mouse	M.La.D.$_{50}$ Standard,[c] Mg./Mouse	Standard Senna Activity, %	Relative Strength of Parts of the Plant
California grown, 1943				
Leaflets	5.4	6.2	115	100
Petioles	8.7	7.2	83	71
Stems	20.4	7.5	37	31
Chattanooga grown, 1943				
Leaflets	7.5	6.1	81	100
Pods	<5.0	<6.3	100+	100+
Petioles	7.2	5.9	82	100
Roots	12.9	6.0	46	57
Chattanooga grown, 1947				
Leaflets	8.7	7.1	82	100
Pods	7.6	8.2	108	130
Petioles	14.2	7.4	52	63
Stems	13.5	7.4	56	68
Tuticorin grown, 1949				
Pods	8.1	5.7	70	...
Imported, stored in Chattanooga 8+ years				
Pods with seeds	9.8	6.9	70	...
Pods with seeds removed	9.2	6.9	75	...

[a] M.La.D.$_{50}$ = Calculated minimum laxative dose in 50% of the mice tested. Three-dose assay, run for three days.
[b] Total number of mice per dose of sample was between 50 and 75.
[c] Total number of mice per dose of standard senna was 30.

In our storeroom we found a box of Tinnevelly pods which was known to have been there for eight years. These pods were tested both with and without the seed. With seed in the pod they showed 70% of standard activity and with the seed removed they showed 75% of the standard activity. By way of comparison, a sample of good green Tinnevelly pods which had been collected in Tuticorin in the summer of 1949[1] showed only 70% of standard senna activity. It appears, therefore, that pods after several years' storage are still an effective laxative.

SUMMARY

1. Tinnevelly senna can be successfully grown to maturity in the Imperial Valley of California and with some risk in other parts of the southern United States. The plant is quickly killed by a temperature drop below 50° F.

2. The leaflets and pods of domestically grown Tinnevelly senna plants have approximately the same laxative potency as those commercially obtained from India and Egypt, and do not differ widely in activity when grown in Tennessee or California.

3. The leaflets and pods of domestically grown Tinnevelly senna are approximately equal in potency, the pods appearing slightly stronger.

4. The petioles of experimentally grown senna are approximately 75 per cent as active as the leaflets from the same plant, the stems and roots are 50 per cent and 55 per cent, respectively.

5. Commercial senna pods, as we have previously reported for the leaves, do not show marked loss of activity upon storage for several years.

[1] Kindly supplied by W. J. Bott, S. B. Penick & Company, New York, N. Y.

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Notes

A Note on the Solubility of Pregnenolone and Pregnenolone Acetate in Various Solvents*

By L. FREEDMAN, E. GREENSPAN, and N. A. LEVIN

INCREASED INTEREST in steroid hormones used in the treatment of chronic diseases has made it necessary to learn something of the solubilities of these compounds in various solvents for methods of purification of the chemicals in manufacturing, as well as for purposes of adapting them for pharmaceutical use.

Solubility data on Δ^5- pregnene-3-*beta*-ol-20-one, known as pregnenolone, one of the steroids now finding use in treatment of rheumatoid arthritis, is not available.

We have accordingly determined the solubility of pregnenolone and pregnenolone acetate in a variety of solvents.

The purity of the compounds used was checked by melting point and specific rotation measurements. For pregnenolone the specific rotation, $[\alpha]_D^{25}$, determined on a solution of ethanol containing 100 mg. hormone per cc., and using a 100-mm. tube was +28°. This falls within the prescribed limits for purity, namely +23° to +33°. The melting point of the pregnenolone was 191–193°.

For pregnenolone acetate the specific rotation, $[\alpha]_D^{25}$, determined on a solution of ethanol containing 100 mg. hormone per cc., and using a 100-mm. tube was +19°. The accepted range for this compound is +15 to +25°. The melting point was 149–151°.

The solvents employed in this study were of reagent grade. Two methods were used in determining solubility. The first was used in the case where the solvent had a boiling point under 100°, and the second in the case where the solvent boiled over 100°. These methods are described below:

1. Excess hormone was refluxed with solvent on a steam bath for one-half hour. After cooling to 25° and allowing solution to remain at this temperature for three hours, 1 cc. of solution was withdrawn with a pipette and evaporated to dryness in a tared dish and weighed. Results were calculated as Gm. of hormone per cc. of solution and are shown in Table I.

2. A weighed amount of hormone, in excess, was heated at 95° with a known volume of solvent for one-half hour. After cooling to 25° and allowing the solution to remain at this temperature for three hours, the residue was filtered on a tared filter. The quantity of dissolved hormone was calculated by difference. The results were calculated as Gm. of hormone per cc. of solvent, and are shown in Table II.

TABLE I

Solvent	Gm. Hormone per Cc. Solution	
	Pregnenolone	Pregnenolone Acetate
Carbon tetrachloride	0.005	0.050
Petroleum ether	0.001	0.010
Ethyl acetate	0.011	0.079
Acetone	0.006	0.027
Chloroform	0.170	0.550
Ethyl alcohol	0.019	0.025
Benzene	0.009	0.260
Isopropanol	0.015	0.020

TABLE II

Solvent	Gm. Hormone per Cc. Solvent	
	Pregnenolone	Pregnenolone Acetate
Propylene glycol	0.001	0.001
Dioxane	0.031	0.202
Benzyl alcohol	0.081	0.111
Benzyl benzoate	Insol.	0.091

Both pregnenolone and pregnenolone acetate show limited solubility in most of the solvents tested, with the exception of chloroform. The acetate derivative is more soluble than the parent moiety.

* Received June 15, 1950, from the Casimir Funk Laboratories, affiliate of U. S. Vitamin Corporation.

A Note on the "Depot" Effect of the Streptomycin Salt of Insulin*

By ROBERT D. BARNARD and ABRAHAM N. SAPERSTEIN

VARIOUS expedients for prolonging the effect of injected insulin have been reported. Oil emulsions (1), relatively insoluble tannates (2), and metallo-derivatives (3), as well as alum and methenamine conjugates (4, 5), have been described. Ferro- and ferribemochromogens of insulin have been used in animal experiments (6), but extensive clinical work appears to have been confined to the commonly used protamine (7), and globin (8) derivatives of insulin.

It was noticed that when ordinary commercial insulin solution (Iletin, Lilly, 80 units per cc.) and streptomycin or dihydrostreptomycin sulfate solution[1] were mixed, a turbidity developed which proved not to be due to the iso-electric precipitation but to the formation of a relatively insoluble streptomycin insulinate. Such a constitution for the precipitate was inferred from its continued insolubility after bringing the mother liquor to the electrometrically determined (glass-electrode) *p*H of 9.5 by the cautious addition of 0.1 *M* NaOH. The constitution was affirmed by the direct formation of a similar salt on addition of a solution of dihydrostreptomycin base to one of sodium insulinate, the latter prepared by dissolving crystalline zinc insulin in 0.02 *M* NaOH. The washed precipitate of dihydrostreptomycin insulinate was found to lower the blood sugar of rabbits injected with a suspension, and the presence of the antibiotic in the precipitate was demonstrated by its inhibitory effect on *Escherichia coli* growth as well as by the development of the orange chromogen reaction in a specimen of the moist precipitate kept for a year at room temperature.

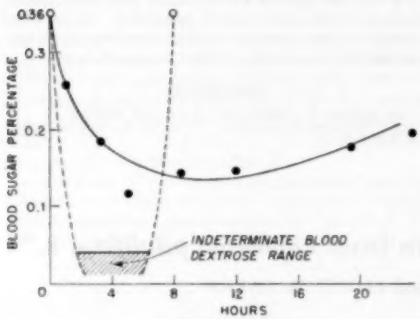

Figure 1

○- - ○ Theoretically derived blood sugar curve of glycogen-depleted diabetic subject receiving 50 units of crystalline insulin.

●— ● Experimentally determined blood sugar curve of presumably glycogen-depleted diabetic subject, S. B., receiving 50 units (in terms of insulin) of insulin-streptomycin.

Because of the possible clinical and pharmaceutical utilities of the fact that streptomycin insulin suspension, as extemporaneously prepared from the marketed forms of the respective constituents, is in such a state of fine dispersion that it may be given with a syringe through a 24-gauge hypodermic needle, the result of such an administration is reported. Figure 1 depicts the protracted insulin effect in a diabetic patient who received no alimentation for twenty-four hours prior to, and during, the observation period following the subcutaneous administration of a mixture of 50 units of insulin (Iletin) and 1 cc. of a 10% solution of streptomycin sulfate. The ensuing blood sugar curve is comparable to that procured after administration of the protamine or globin conjugates.

Potential repository properties of the insulin salt of streptomycin might have been anticipated from other similarities of the latter to protamine or globin. All are strong bases of relatively high molecular weight. Streptomycin, like protamine (9) and globin (10), forms an insoluble heparinate (11).

While it is doubtful if ordinary therapeutic administration of streptomycin would interfere with the systemic effect of insulin given concurrently, the matter might still profitably be subjected to observation in patients receiving both medications. Such observation might also be directed to theoretically possible potentiation of insulin effect by streptomycin, since the latter is a guanidyl derivative and some of the latter have pronounced hypoglycemic effects. However, it would appear from our preliminary chemical studies that local streptomycin concentrations required to precipitate insulin in the form of the streptomycin salt, *in vivo*, would be far greater than are ever extant in the blood and tissues, except possibly in patients receiving intensive, protracted streptomycin dosage during renal failure. Perhaps the only importance of the observation reported is that the streptomycin derivative of insulin might be useful as a repository in patients who evinced sensitivity to the protamine or globin conjugates.

* Received November 14, 1950, from the Medical and Radiologic Services, Terrace Heights Hospital, Hollis, L. I., N. Y.

[1] The streptomycin salts used in this investigation were generously supplied by Chas. Pfizer and Co., Brooklyn, N. Y.

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A Note on the Determination of Purity of Homatropine Methyl Bromide*

By RICHARD H. ROBINSON, HELEN C. KAULFERS, and ELEANOR B. DUELLY

HOMATROPINE METHYL BROMIDE (H. M. B.) can be assayed for purity with a nitrogen or halogen determination but these methods are not very specific. Reimers (1) describes a method of extracting the mandelic acid from the hydrolysis products of H. M. B. with ether. The A. O. A. C. (2) method of extraction, using mixed chloroform–ether solvent, eliminates most of the disadvantages of using ether alone. This combined procedure, with certain small changes, has been used in this laboratory to check the purity of bulk H. M. B.

EXPERIMENTAL

One gram of H. M. B. is dissolved in 5 ml. of 0.5 *N* aqueous sodium hydroxide, contained in a 250-ml. glass-stoppered separatory funnel. After standing for at least thirty minutes, the liquid is neutralized and made acid with an excess of concentrated hydrochloric acid. Sufficient solid sodium chloride to completely saturate the water present is added and dissolved. The mandelic acid is then extracted with at least six 30-ml. portions of extracting solvent (three parts of chloroform plus one part of ethyl ether). The combined extract is washed with 5 ml. of saturated sodium chloride and then filtered through cotton into a weighed beaker. The solvent is evaporated at a temperature not to exceed 35° and the beaker is reweighed. The gain in weight is mandelic acid and can be multiplied by the factor 2.435 to yield the H. M. B. originally present. The melting point of the isolated mandelic acid should be 117 to 119° (corr.).

Determinations made in this manner by three analysts yielded between 99.5 and 100.5% recoveries of the mandelic acid from H. M. B. Melting point determinations on each residue were within one degree of the recorded melting point of pure mandelic acid (118.1°). Extracts of unhydrolyzed H. M. B. yielded no significant residue on evaporation. The isolated mandelic acid was also identified by the odor of benzaldehyde which it produces on periodic acid oxidation.

SUMMARY

A method specific for mandelic acid from homatropine methyl bromide is presented. It is more specific for homatropine methyl bromide than existing methods and is of a similar degree of accuracy.

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* Received November 6, 1950, from the Malthie Laboratories, Inc., Newark, N. J.

A Note on the Isolation of Quercitrin from *Euphorbia pilulifera*, L.*,†

By FLOYD P. HALLETT‡ and LLOYD M. PARKS

WHILE the isolation of quercetin (3,3′,4′,5,7-pentahydroxyflavone) from a number of plants has been reported, its glycoside, quercitrin (quercetin-3-rhamnoside), has been reported much less frequently. Power and Browning (1) isolated quercetin from acidified aqueous solutions of the alcohol extract of *Euphorbia pilulifera*, L., but did not determine whether it existed in the plant in the free state or as the glycoside which was hydrolyzed in the acid solution. In the course of work on isolation of the antispasmodic principle of this plant (2) we have obtained the glycoside, quercitrin, by ether extraction of the aqueous solution of a hydroalcoholic extract of the drug.

EXPERIMENTAL

Finely ground *E. pilulifera* (400 Gm.) was used to prepare a fluidextract according to the N. F. VIII Process A, with diluted alcohol. The alcohol was removed by distillation at 60° *in vacuo*, the residue made up to 150 cc. with water, the water-insoluble material removed by filtration, and the aqueous filtrate was extracted for thirty hours with ether in a continuous extractor.

Microcrystalline yellow platelets separated from the ether extract when it was allowed to concentrate by evaporation. This material was purified by washing with warm ethyl acetate, followed by five recrystallizations from water, and was identified as quercitrin by its melting point, absorption spectrum, and by hydrolysis to quercetin.

For purpose of comparison, quercitrin was prepared from lemon flavin by the method of Morrow and Sandstrom (3). When dried at 100° for two hours, both the quercitrin from *E. pilulifera* and that from flavin melted at 179–180° and gave no depression when mixed. Previously reported melting points for the compound vary from 168° (4) to 182–185° (5). The absorption curves for both compounds, in 95% alcohol, were nearly identical (Fig. 1), both having maxima at 2,570 and 3,530 Å. Marchlewski and Skarzynski (6) report maxima for quercitrin at 2,612 and 3,560 Å., while Guthrie, *et al.*, (7) report them at 2,600 and 3,600 Å.

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† Supported in part by a research grant from Templetons, Ltd., Toronto, Canada, and Templetons, Inc., Buffalo, N. Y.
‡ American Foundation for Pharmaceutical Education Fellow.

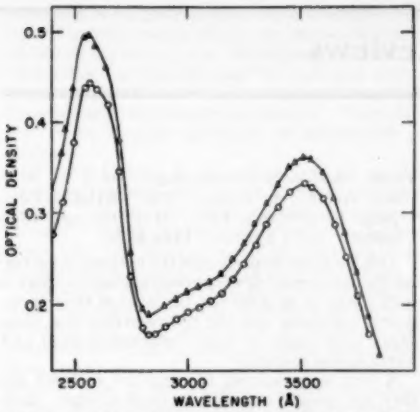

Fig. 1.—Absorption spectra of quercitrin from flavin (-○-○-○-) and from *Euphorbia pilulifera* (-△-△-△-).

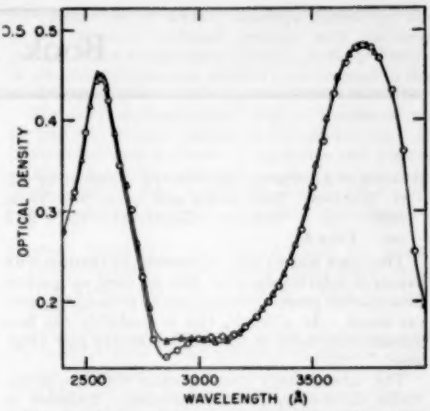

Fig. 2.—Absorption spectra of quercetin from flavin quercitrin (-○-○-○-) and from *Euphorbia pilulifera* quercitrin (-△-△-△-).

A sample of quercitrin (0.25 Gm.) from *E. pilulifera* was hydrolyzed by heating for one hour on the steam bath in 10 cc. of water with 20 drops of dilute HCl. After recrystallization from 80% alcohol, the aglycon was dried at 120° and melted at 310–314°. Its absorption curve (maxima at 2,560 and 3,730 Å.) was practically identical with that of quercetin (maxima at 2,570 and 3,720 Å.), which was prepared in the same way from the quercitrin of flavin (Fig. 2). Guthrie, *et al.* (7), report maxima for quercetin at 2,560 and 3,720 Å.

Acetylation of the aglycon of quercitrin from *E. pilulifera* with acetic anhydride and sodium acetate yielded quercetin pentaacetate, m. p. 195.5 to 197°, absorption maxima at 2,520 and 2,990 Å. Literature reports this compound melting at 194–196° (1) and at 200° (7), with absorption maxima at 2,530 and 2,990 Å. (7).

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BOOKS RECEIVED

Carotenoids. By PAUL KARRER and ERNST JUCKER. Translated and revised by ERNEST A. BRAUDE. Elsevier Publishing Co., Inc., New York, 1950. x + 384 pp. 17 x 24.5 cm. Price $8.50.

The Story of California Pharmacy. By GEORGE GRIFFENHAGEN. Published in cooperation with the Pacific Drug Review by the American Institute of the History of Pharmacy, Madison, Wis., 1950. 58 pp. Illustrated. 14 x 21 cm. Price $1.

Chemistry and Biology of Proteins. By FELIX HAUROWITZ. Academic Press, Inc., New York, 1950. xii + 374 pp. Illustrated. 15.5 x 23.5 cm. Price $5.50.

Laboratory Manual for Pharmacognosy. 2nd ed. By EDWARD P. CLAUS. C. V. Mosby Company, St. Louis, 1950. 111 pp. + study outline pages. 21.5 x 28 cm. Price $3.25.

Drugs from Plants. By TREVOR ILLTYD WILLIAMS. Interscience Publishers, Inc., New York, 1950. 119 pp. Illustrated. 12.5 x 19 cm. Price $1.25.

Ion Exchange Resins. By ROBERT KUNIN and ROBERT J. MYERS. John Wiley and Sons, Inc., New York, 1950. xi + 212 pp. Illustrated. 15 x 23.8 cm. Price $4.75.

The Dispensatory of the United States of America. 1950 Edition. Vol. 2. New Drug Developments Volume. ARTHUR OSOL, Editor-in-Chief. J. B. Lippincott Company, Philadelphia, 1950. 128 pp. 18 x 27 cm.

Methods in Medical Research. Vol. 3. RALPH W. GERARD, Editor-in-Chief. Year Book Publishers, Inc., Chicago, 1950. xii + 312 pp. Illustrated. 14 x 22.5 cm. Price $7.

1950 Proceedings of the National Association of Boards of Pharmacy, Inc. Chicago, 1950. Forty-Sixth Annual Convention, held at Atlantic City, N. J., May 2–3, 1950. 210 pp. 15.3 x 23 cm.

Soybeans and Soybean Products. Vol. I. Edited by KLARE S. MARKLEY. Interscience Publishers, Inc., New York, 1950. xvi + 540 pp. Illustrated. 15.5 x 24 cm. Price $11.

Book Reviews

Analytical Absorption Spectroscopy. Edited by M. G. MELLON. John Wiley and Sons, New York, 1950. vii + 618 pp. Illustrated. 15 x 23.5 cm. Price $9.

This book was written to furnish the chemist with practical information that will be used in spectrophotometric procedures and not to present theoretical detail. As a result, this is probably the best introductory book to spectrophotometry now available.

The introductory chapter deals with the preparation of samples for measurement. Included in this part are details of techniques required for separation, removal of interfering substances, concentration, development of color, and compensation for interfering absorbancies. Although it is obvious that this chapter cannot give a complete literature survey, it contains suggestions and general procedures which will be useful in the majority of spectrophotometric methods. This section of the book will be of particular value to the worker who is developing new assay procedures. The thoroughness of the treatment of this subject is indicated by the fact that 574 references are included.

The second section of the book develops the general principles of spectrophotography and furnishes the necessary theoretical background such as Bouguer-Beer's law. Deviations from Beer's law with reasons and methods for compensating are discussed. The use of various types of curves and their value is presented.

A separate section is devoted to Color Comparimeters, which points out the applicability of these instruments and the sources of error noted in their use.

Filter Photometers and their advantages and disadvantages are treated in some detail. Following this, a large section on spectrophotometers is presented, this part of the book being divided according to type of instrument: Spectrophotometers for Ultraviolet and Visible Regions, Photographic Methods, Application of Ultraviolet and Visual Spectrophotometric Data, and Spectrophotometers for the Infrared Region. The section on the application of spectrophotometric data is particularly worthy of note since it presents in clear, logical form the direct use of various types of measurements for qualitative and quantitative determinations. The various phenomena which explain deviatious are presented, and control or correction of these deviations is discussed. Spectrophotometry of mixtures is also presented. A large number of examples are used to illustrate the principles discussed in the text.

A final section of the book discusses the measurement and specification of color, a field which is becoming particularly important since instruments for measurement of color have been developed and are becoming more widely used in industry. Methods of color specification are included, along with a description of instruments and charts used in color measurements.

Recent Advances in Pharmacology. By J. M. ROBSON and C. A. KEELE. The Blakiston Company, Philadelphia, 1950. xi + 418 pp. Illustrated. 13.5 x 20.5 cm. Price $5.50.

This book was written with the purpose of surveying the more recent developments in pharmacology in such a way as to point out the basis of their therapeutic usefulness and the contributions that these drugs have made to basic pharmacological and physiological concepts.

A very large number of drugs are included and they are usually classified in small groups. Anticholinesterase Drugs includes a brief discussion of the sites of action of the different drugs of this group. The mode of action and the effect in various diseases of DFP, HETP, and TEPP are presented, and conclusions are drawn as to their value in medical practice. DFP is recommended for glaucoma and it is stated that the value of this group of phosphates in other diseases, such as myasthenia gravis, is still questionable. The effects that these drugs have on other systems of the body are presented in brief form. The curariform drugs are discussed as to their mode of action and their effects on circulation, respiration, and the nervous system. The use of curare and the synthetic curariform agents in anesthesia, hyperkinetic states, etc., is presented along with summaries of results that have been obtained. Tetraethylammonium salts and Myanesin are included in this group.

There is an excellent chapter on antihistamine drugs which presents the theories of mechanism of action, therapeutic usefulness and administration and dosage of these compounds.

Other sections of the book cover such subjects as antithyroid drugs, antibiotics, antimalarials, analgesics, and the newer vitamins. Also, there are short sections on the newer methods of pharmacology, which include the newer types of studies used to determine mode of action and to show absorption and excretion.

This book does not present its subjects in any great detail but it does cover most of the important developments in pharmacology of recent years. It should serve very well as a refresher course for those who have not been able to keep up with the more recent developments in pharmacology and therapeutics, and should be especially valuable to the practicing physician.

Die Tablettenfabrikation und ihre maschinellen Hilfsmittel. 5th ed. BY JOHANNES ARENDS. Springer-Verlag, Berlin, 1950. 262 pp. Illustrated. 15 x 21 cm. Price DM 25.50.

Although this is a fourth revision, this book is probably not well known in this country, and the reviewer believes it is worthwhile to summarize the content.

This book is directed toward presenting a set of useful formulas for tablets, rather than giving in-

structions in the general procedures of tablet making. The first portion of the text consists of a brief historical review of the development of tablet medication. It then proceeds to short and simple discussions of the methods of granulation, precompressing, and compressing tablets. Very little is given on coating techniques or composition of coatings.

Of special value is the description of various European machines used for granulating and compressing. Photographs of most of the machines are included. Research laboratories, university laboratories, and retail pharmacies will be interested to see the machines designed for small-scale operations.

The second section of this book, comprising 190 of the 262 pages of the text, contains formulas with directions and brief discussions of various tablet compositions. The formulas are listed according to alphabetical order of the active or principal component. The group includes tablets containing simple chemical mixtures, crude drugs, extracts, and alkaloids. Also, formulas for effervescent tablets, compressed lozenges, soluble tablets and pastilles are given. Tablets of homeopathic strength are also described. The list of types of medicinals and mixtures is so extensive as to furnish examples for almost any conceivable type of tablet composition.

Much of the text is directed toward manufacture of small lots of tablets. Directions and formulas are given for several basic granulations to which can be added almost any active ingredient by simple mixing, so that suitable tablets can be compressed from the mixture. This type of formulation would be most useful to the pharmacist who may make a few tablets at a time.

Carotenoids. By PAUL KARRER and ERNST JUCKER. Translated and revised by ERNEST A. BRAUDE. Elsevier Publishing Co., Inc., New York, 1950. x + 384 pp. 17 x 24.5 cm. Price $8.50.

The authors, in their foreword to this book, state: "It was the desire to sift and collate the extensive literature on carotenoids which led to the writing of the present monograph on this class of natural pigments."

Thirty years ago little was known concerning the chemistry of the carotenoids and knowledge concerning them was confined, for the most part, to their distribution and certain information concerning their biological significance. In the period since 1920 great advances have been made in determining the chemical nature of the polyene pigments and in elucidating their biological significance.

The authors have been successful indeed in their effort to sift and collate the extensive literature on carotenoids that has developed during the past two or three decades. The book is logically arranged in two parts. In the first part, two short chapters cover the mode of occurrence, detection and estimation, and the formation and physiological significance in plants of the carotenoid pigments. In the next seven chapters the isolation, chemical constitution, *cis-trans* isomerism, methods of determining the constitution, relationships between the constitution and color, synthesis, and distribution in nature of the carotenoids are summarized and discussed.

In a special part of the book, the carotenoid hydrocarbons of known constitution are listed and discussed in one chapter. This is followed by chapters on carotenoids of known structure containing hydroxyl groups, carbonyl groups, and carboxyl groups. The carotenoids of only partially known or completely unknown structure are covered in the final chapter.

The book is documented with references to all pertinent literature relating to the carotenoids. It is provided with an index of vegetable and animal sources of carotenoids and it has an excellent subject index. The book is designed to serve anyone interested in any phase of the field of carotenoids.

Organic Syntheses. Vol. 30. ARTHUR C. COPE, Editor-in-Chief. John Wiley and Sons, Inc., New York, 1950. vi + 115 pp. 15 x 23.5 cm. Price $2.50.

This new volume contains 39 new syntheses, and the familiar style of the previous volumes is maintained. Perhaps of special interest to pharmaceutical chemists will be the methods for 3-aminopyridine, *dl*-aspartic acid, benzoylcholine iodide and chloride, ethylenimine, ethyl phenylcyanoacetate, *o*-nitroacetophenone, and vanillic acid.

The method for preparation of *dl*-aspartic acid is a synthetic one which utilizes diethyl phthalimidomalonate and ethyl chloroacetate as starting material.

Ethylenimine is prepared from β-aminoethylsulfuric acid by treatment in 40% sodium hydroxide to obtain a yield of purified material equal to 34–37%. This reaction is particularly useful since the beginning material is commercially available.

o-Nitroacetophenone is prepared by reacting magnesium ethoxide with malonic ester and the resulting product with *o*-nitrobenzoyl chloride. A yield of 82–83% is obtained.

Two procedures for the preparation of vanillic acid are described, both using vanillin. In the first procedure the vanillin is oxidized with freshly precipitated silver oxide, and in the second instance vanillin is fused with potassium hydroxide. The yields are high for both methods.

As has been the practice in the past, this volume of organized syntheses presents notes which are useful in preparation of intermediates, or which indicate commercial availability of intermediates. Reference to other methods of preparation are given, as well as to the original publication describing the method adopted by *Organic Syntheses*. Notes are made to any special precautions which should be used in handling the chemicals for a given synthesis.

Advanced Organic Chemistry. By REYNOLD C. FUSON. John Wiley and Sons, Inc., New York, 1950. x + 669 pp. 15 x 23.5 cm. Price $8.

This text is intended to furnish the student who has already had a course in general organic chemistry with a more thorough knowledge of fundamental concepts of organic reactions and theory. It does not go into as great detail as most advanced texts on each type of reaction, and has eliminated many exceptions to reactions commonly met with in such texts. Subject matter is arranged according to reaction rather than structure. Such groupings as Oxidation Reactions, Hydrogenation and Hydrogenolysis and Reactions of Active Methylene Groups are found.

Though this type of organization might be questioned at first glance, after some study of the book it becomes apparent that it is a most satisfactory and logical method of presentation and it seems likely that it will prove understandable and stimulating to the student.

The author brings out in the text the concept of electron density as the basis of organic reaction and illustrates electrophilic or nucleophilic attack. The physical concepts underlying this theory are not presented, but it is assumed that such information would be obtained by the student in the later and more advanced course. There are many instances where electric interpretations of reactions might be used but are not presented.

In accordance with his intention, the author has not included many of the newer and less commonly used reagents, though the text is quite complete in regard to the better-known reagents and reactions.

This book should prove most acceptable to teachers of advanced organic chemistry, as well as to their students. It is not so advanced as to be above use by undergraduate students.

Chemistry and Biology of Proteins. By FELIX HAUROWITZ. Academic Press, Inc., New York, 1950. xii + 374 pp. Illustrated. 15.5 x 23.5 cm. Price $5.50.

The aim of the author has been to design a textbook suitable for use by graduate students in chemistry and in the biological sciences in which the fundamental aspects of protein chemistry are stressed. He has apparently accomplished this aim in a highly acceptable manner by limiting the topics to a concise but clear discussion of protein structure, biological activity of proteins, and biosynthesis of proteins in the living cell.

Considerable space is devoted to enzymes, hormones, and immunologically active proteins. The isolation and analysis of proteins, their amino acid content, and their physical and chemical properties are, however, adequately covered.

In addition, views on the structure of native and denatured proteins and the mechanism of protein synthesis in the living cell are presented in an entirely acceptable manner.

While the author is entirely competent to write authoritatively on the subject of proteins, he has attempted to substantiate each statement by references to original sources of information. In order to accomplish this, more than 1,500 original papers are cited. This feature, while unusual in a textbook, serves to enhance its value to research workers having occasion to make use of published references to the chemistry and biology of proteins.

The subject index is adequate, but no author index is provided. The typography of the book is excellent and results in a finished product that is conducive to ease in reading.

The Story of California Pharmacy. By GEORGE GRIFFENHAGEN. Published in cooperation with the Pacific Drug Review by the American Institute of the History of Pharmacy, Madison, Wis., 1950. 58 pp. Illustrated. 14 x 21 cm. Price $1.

The Story of California Pharmacy is pleasant reading. Prompted by the centennial anniversary of California's statehood, Mr. Griffenhagen has produced a well-organized and well-written work, which includes not only the last century of California pharmacy, but an interesting account of pharmacy as practiced by the Indians and, somewhat later, by the Spaniards.

The discovery of gold in 1848 was, of course, responsible for a sudden, mass migration to California. The subsequent impact on pharmacy, the real birth of modern pharmacy in California, is depicted well. The growth of pharmacy is traced by accounts of pioneer pharmacists, the beginnings of pharmaceutical associations and publications, the establishment of the first wholesale drug houses, and the advent of the chain store systems.

A heartening note to historians and students of history is contained in the foreword written by Dr. George Urdang, Director of the American Institute of the History of Pharmacy, who states that "the Institute has decided to . . . initiate a series of [similar] booklets until all states of the Union have thus been covered."

Colloidal Dispersions. By EARL K. FISCHER. John Wiley and Sons, Inc., New York, 1950. vii + 387 pp. Illustrated. 15.5 x 24 cm. Price $7.50.

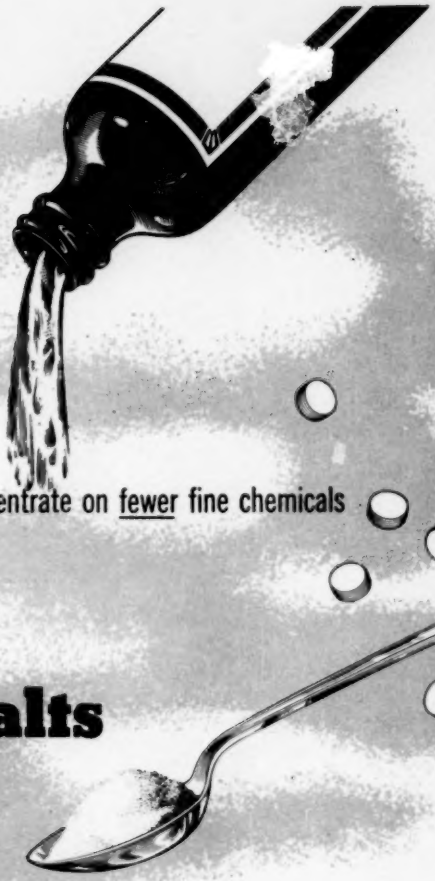
In Dr. Fischer's book both the theoretical and practical considerations of colloidal dispersions are adequately covered. The first five chapters present the theoretical background and discuss, among other topics, particle size, the solid-liquid interface and wetting, the state of the dispersed solid, and rheological properties of dispersions.

The practical aspects of the production and stabilizing of dispersions are treated in chapters on surface-active agents, the process of comminution, mixing, the operating of different types of mills (roll, ball and pebble, disk, cone and colloid).

Of special interest is a discussion on the dispersion of solids in nonaqueous vehicles in a chapter on dispersion by phase transfer—the flushing process.

The book should be of pharmaceutical interest to teachers and to those engaged in the development of dosage forms for drugs that can be most satisfactorily prepared, stabilized, and used in the form of dispersions.

The book is well-documented throughout, and is provided with an author index and a really satisfactory subject index. The printing and binding are of excellent quality.



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